

# Bulletin of the Agricultural Chemical Society of Japan.

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## *The Agricultural Chemical Society of Japan.*

President : Kintaro OHSHIMA.

The Council of the Agr. Chem. Soc. of Japan has decided to publish English Abstract of those papers appearing in the Journal in a separate form in order to facilitate the circulation in foreign countries.

Bulletin of the Agr. Chem. Soc. of Japan is published for this purpose from May 1926 monthly. The numbering begins with Vol. 2, No. 5. The earlier parts are represented by the English abstracts published in the Journal annexed to the Japanese texts.

The articles to be appeared in the Bulletin must be concise, supplied with experimental methods and data and understandable, without specially referring to the Japanese texts. It ought, however, not exceed four printed pages as a rule. Any longer articles may be accepted according to the decision of the Council, with or without charge for exceeding pages.

Journal of the Agr. Chem. Soc. of Japan will be published in Japanese as formerly. Those desiring the detailed information of the articles appeared in the Bulletin may look for in the Journal of the same Number or the same Volume.

Editor : Kintaro OHSHIMA.

Associate Editors : Kakuji GOTŌ and Yoshikazu SAHASHI.

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## Carotin in Mango Fruit (*Mangifera indica* Lin.).

By

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(Agricultural Chemical Department, Taihoku Imperial University, Taiwan Japan.)

(Received August 16, 1932.)

Works of H. Euler, P. Karrer<sup>(1)(2)(3)</sup>, K. Kawakami<sup>(4)</sup>, H. Mattil<sup>(5)</sup>, R. Kuhn<sup>(6)</sup>, and their co-workers to the effect that carotin has a function of vitamin A gave the significant contribution in the study of food chemistry. The colouring matter of water-melon<sup>(7)</sup> and tomato<sup>(8)</sup> has more or less to do with carotin, so that the occurrence of carotin in other fruits which contain yellow pigment might be reasonably suspected. The fruit pulp of mango is of well-known yellow colour, and if this pigment belongs to carotin, it may give interesting information to the nutritive value of mango fruit.

Mango fruit, we have got, was the native variety produced in Formosa. According to M. Isii the fresh pulp of the fruit contains 81.7% water, 3.0% citric acid, 1.5% glucose, 4.9% fructose, 5.5% sucrose, 0.5% crude fiber, 1.3% ether extract, 0.1% nitrogen, and 0.5% ash.

The yellow pigment is easily soluble in ether, pet. ether, chloroform, and partly soluble in alcohol, but not in water. The colour reaction by antimony trichloride is blue. These behaviours suggested the existence of a certain carotinoid. The isolation was carried out according to Willstätter, Kuhn and Karrer.

Main obstacles for isolation were the presence of waxy substance mixed with the pigment and moreover, as the fruits are produced only in hot summer time, the treatment of a large amount at one time was somewhat cumbersome. The details of the experiment will be found in the experimental part. We could isolate pure carotin in crystalline form, but the complete separation<sup>(9)</sup> of  $\alpha$ - and  $\beta$ - carotin were not attempted owing to the paucity of the material; only the general separation was carried out by the fractional crystallization from carbon disulphide, ethyl alcohol and pet. ether.

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(1) Helv. Chim. Acta, (1929), 278.

(2) Helv. Chim. Acta, (1931), 831.

(3) Helv. Chim. Acta, (1931), 1036, 1431.

(4) Proc. Imp. Acad. Jap., (1929), 213.

(5) J. Biol. Chem., (1931), 1050.

(6) B., **64** (1931), 1859.

(7) B., **63** (1930), 2881.

(8) Helv. Chim. Acta, (1931), 154.

(9) Hoppe Sy. Z. Physiol. Chem., **200** (1931), 246.

The main fraction melted at  $179^{\circ}$  and specific rotation was  $[\alpha]_D^{25} = +136^{\circ}$ . These indicated the fraction to be a mixture of  $\alpha$  and  $\beta$  carotin, the absorption spectrum also proving it. In addition to this main fraction a little quantity of crystal, melting at  $174^{\circ}$  was separated. It was more soluble in pet. ether and was ascertained to be  $\alpha$ -carotin. The yield of main fraction was 0.06 g. from 38 kg. of fresh mango fruit.

A quantitative estimation of carotinoids in mango fruit was determined according to Kuhn and Brockmann<sup>(10)</sup>. The result was 0.1179 g. carotin, 0.0785 g. xanthophyll in ester form, and 0.0156 g. free xanthophyll, in 1 kg. anhydrous pulp of mango fruit.

The isolation of xanthophyll in crystalline form was unsuccessful, so it was determined by absorption spectrum, and the result coincided with the blattxanthophyll<sup>(11)</sup>.

It seems from the experiment that the content of carotinoids is varied by the variety and the ripeness of the fruit.

The physiological action of the carotin as vitamin A was determined by the feeding experiment which was kindly carried out by K. Kawakami in the Institute of Physical and Chemical Research in Tokyo.

The daily doses of 0.05 mg. of the carotin (m. p.  $179^{\circ}$ ) was quite sufficient for curing of albino rats from their deficiency of vitamin A.

### Experimental\*.

#### *Isolation of carotin:*

The fresh pulp of mango fruit (38 kg.) was mashed, and extracted with ether three times. The ether was then washed with water, dried and distilled in the atmosphere of carbon dioxide. When the ether was evaporated completely by vacuum, a yellowish red oily substance was obtained. This was then dissolved in pet. ether, washed by 90% methyl alcohol several times to get rid of xanthophyll.

When the pet. ether solution was dried with sodium sulphate, evaporated to a small quantity and left in an ice chest for 48 hours, it subsided colourless needles mixed with waxy substance. The filtrate from the above was then saponified by 2 *N* alcoholic potash in nitrogen atmosphere on water-bath during two hours. By this treatment, carotin crystallized out slowly, which was collected and washed first with water and then with methyl alcohol. The crude carotin thus obtained was dissolved in a little quantity of carbon disulphide and by the addition of absolute alcohol fractionally precipitated.

(10) Hoppe Sy. Z. Physiol. Chem., **206** (1932), 41.

(11) " " " " " **197** (1931), 141.

\* The experiment was carried out in each summer of 1930 to 1932.

When the filtrate from the first crop was cooled in an ice chest a second fraction was obtained, and third fraction was separated by adding absolute alcohol to the above filtrate. The each fraction was recrystallized from pet. ether. We could, thus, separate two kinds of crystal of different melting point. The main fraction melted at  $179^{\circ}$ , and a very small part melted at  $174^{\circ}$ , both of which showed a deep red metallic lustre, the former being long prisms and the latter rhombic plates.

*Analysis of carotin:*

1) Carotin m. p.  $173-174^{\circ}$ .

Substance (mg.)	CO <sub>2</sub> (mg.)	H <sub>2</sub> O (mg.)	C %	H %
1.891	6.203	1.791	89.46	10.60
1.090	3.583	1.033	89.65	10.61
Calc. for C <sub>40</sub> H <sub>56</sub>			89.55	10.45

2) Carotin m. p.  $179^{\circ}$ .

Substance (mg.)	CO <sub>2</sub> (mg.)	H <sub>2</sub> O (mg.)	C %	H %
1.436	4.699	1.340	89.25	10.44
1.834	12.548	3.535	89.26	10.32
Calc. for C <sub>40</sub> H <sub>56</sub>			89.55	10.45

*Absorption spectrum:*

1) Absorption spectrum of  $\alpha$ -carotin (m. p.  $173-174^{\circ}$ ) by quartz, spectrograph in chloroform solution, absorption bands;

I 452—487  $\mu\mu$ ,                      II 495—507  $\mu\mu$ .

2) For the main fraction (m. p.  $179^{\circ}$ ) the maximum absorption was observed by spectro-photometer in CS<sub>2</sub> and chloroform solution.

Absorption bands

	I	II	III
In CS <sub>2</sub>	449 $\mu\mu$	475 $\mu\mu$	514 $\mu\mu$
In chloroform	435	462	487

*Specific rotation:*

The optical rotation of the main fraction m. p.  $179^{\circ}$  in CS<sub>2</sub>;

$$[\alpha]_{\text{C}}^{25} = (+0.15 \times 100) : (1.5 \times 0.7232) = + 135^{\circ}.$$

*Estimation of carotinoids in mango fruit:*

320 g. fresh pulp from 574 g. fruit (five) was mashed and from this homogeneous mass 60 g. was taken and treated with methanol and then methanol pet. ether mixture, until all the pigment was disappeared. Then diluted with water and extracted by pet. ether, filled up to 500 c.c.. From

this solution 100 c.c. was taken and estimated carotinoids according to Kuhn and Brockmann<sup>(10)</sup>.

Mean reading of carotin solution corresponds to 10 mm.

standard solution	.....	4.6 mm.	.....	total solution	250 c.c.
"	"	free xanthophyll	"	"	"
standard solution	.....	15.5 mm.	.....	total solution	100 c.c.
"	"	ester form xanthophyll	"	"	"
standard solution	.....	7.7 mm.	.....	total solution	250 c.c.

Carotin in 12 g. fresh pulp

$$= 0.0047 \times 10/4.6 \times 250 = 0.245 \text{ mg.},$$

free xanthophyll in 12 g. fresh pulp

$$= 0.000504 \times 10/15.5 \times 100 = 0.0325 \text{ mg.},$$

ester form xanthophyll in 12 g. fresh pulp

$$= 0.000504 \times 10/7.7 \times 250 = 0.163 \text{ mg.}$$

	Carotinoids in 1 kg. pulp mango fruit	
	Fresh pulp (water content 82.7%)	Anhydrous pulp
Carotin	20.41 mg.	0.1178 g.
Free xanthophyll	2.70	0.0156
Ester xanthophyll	13.58	0.0780

Absorption maximum of xanthophyll fraction in CS<sub>2</sub> by spectrophotometer.

#### Absorption bands

	I	II	III
Free xanthophyll	506 $\mu\mu$	473 $\mu\mu$	446 $\mu\mu$
Ester form xanthophyll	506—505	475	445

## On the Carotinoids in Fresh Tea-Leaf and Fermented Tea.

By

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(Received August 8, 1932.)

The colouring matters of fermented tea such as black and Oolong tea

consist of water soluble and insoluble parts, and the former is mainly tannic material and the latter is suggested to be carotinoids and chlorophyll. In addition to the study of tannin in fresh tea-leaf and fermented tea, the preparation of which is now carried on in our laboratory, we have investigated the carotinoids.

The materials, we have taken, were Formosan tea-leaf and its fermented tea. The fresh tea-leaves, and fermented tea-leaves, which were taken from fermenting room in the factory, were dried at 40° and powdered. This material was extracted with ether, the ethereal solution was then concentrated and saponified with alcoholic potash; this was diluted with much water and again extracted by ether, which was then washed with water, dried and evaporated ether completely. The red colouring matter thus obtained was separated into carotin and xanthophyll by means of pet. ether and 90% methyl alcohol.

#### *Xanthophyll.*

The methyl alcoholic solution separated from pet. ether, was diluted with water until 70% concentration, shaken with pet. ether, and left standing in an ice-chest. After 24 hours, xanthophyll crystallized out between two layers. It was dissolved in little chloroform and recrystallized by adding pet. ether, and finally recrystallized several times from methyl alcohol. The purified melting substance melted at 192°. From black tea, Oolong tea and Touchang tea we have separated xanthophyll of the same melting point. The absorption bands of spectrum was on 504  $\mu\mu$ , 473  $\mu\mu$ , 446  $\mu\mu$ , and the specific rotation was  $[\alpha]_D^{20} = +170^\circ$  (in acetic ester). These results as elemental analysis agreed with those of blattxanthophyll<sup>(1)</sup>.

#### *Carotin.*

When the pet. ether solution mentioned above, was evaporated in vacuum, added with absolute alcohol and cooled in an ice chest, carotin was separated. Thrice recrystallized from benzene and methanol, deep red crystals of carotin (m.p. 174°) were obtained. Carotin can also easily be obtained by treating the above pet. ether extract with acetone before the separation of xanthophyll; but such treatment makes the isolation of xanthophyll rather difficult. The maximum absorption of spectrum was on 510—511  $\mu\mu$ , 477  $\mu\mu$ , 435  $\mu\mu$  (in CS<sub>2</sub>). Specific rotation was  $[\alpha]_D^{20} = +370^\circ$  (in CS<sub>2</sub>). These experimental data and the results of elemental analysis have just agreed with  $\alpha$ -carotin of Kuhn<sup>(2)(3)</sup>. We had also isolated carotin in crystalline form from

(1) Kuhn and Winterstein: Hopp. phys. Chem., **197** (1931), 141; **63** (1930), 1489.

(2) Kuhn: Naturwissenschaften, **306** (April) (1931).

(3) Kuhn and Brockmann: " , **64** (1931), 1859.

Oolong tea, Touchang tea, but could not from black tea, so we had confirmed its presence in the latter by the spectrum analysis.

The estimation of carotinoids was carried out by the method of Willstätter and Stoll<sup>(4)</sup>.

The results were :

Carotinoids in 1 kg. materials (anhydrous)

	Carotin (g.)	Xanthophyll (g.)
Fresh leaf	0.1751	0.4544
Fermenting tea-leaf	0.1571	0.4237
Touchang tea	0.1681	0.4257
Oolong tea	0.0784	0.2441
Black tea	0.0715	0.3686
Green tea	0.1571	0.4175

As for carotin in green tea Miss Tsujimura<sup>(5)</sup> has recently isolated it in crystalline form, our experiment had been accomplished before her publication.

*Elemental analysis of carotinoids.*

(1) *Xanthophyll* :

Xanthophyll separated from both fresh leaf and fermented tea had the same melting point 192°. The analytical results were :

Substance (mg.)	H <sub>2</sub> O (mg.)	CO <sub>2</sub> (mg.)	H%	C %
5.304	4.671	16.435	9.86	84.51
2.550	2.211	7.887	9.73	84.35
Calc. C <sub>40</sub> H <sub>56</sub> O <sub>2</sub> required			9.93	84.44

(2) *Carotin* :

Carotin in fresh leaf and fermented tea had the same melting point 174°.

Substance (mg.)	H <sub>2</sub> O (mg.)	CO <sub>2</sub> (mg.)	H%	C %
3.514	3.225	11.436	10.37	88.77
Calc. C <sub>40</sub> H <sub>56</sub> required			10.52	89.48

*Sepecific rotation.*

Xanthophyll m p 192°.

$$\text{in acetic ester} \quad [\alpha]_D^{23} = (+ 0.02^\circ \times 100) : (1 \times 0.0117) = + 170^\circ$$

(4) S. Palmer: Carotinoids and Related Pigment, 248.

(5) Tsujimura: Sc. Pap. I. P. C. R., Tokyo., 349 (1932).

in CS <sub>2</sub> .....	$[\alpha]_D^{20} = (+ 0.04^\circ \times 100) : (1 \times 0.0117) = + 341^\circ$
Carotin m p 174°.	
in CS <sub>2</sub> .....	$[\alpha]_D^{23} = (+ 0.13^\circ \times 100) : (2 \times 0.0184) = + 353^\circ$

*Absorption spectrum.*

The extinction coefficient had been measured using Adam-Hilger's spectro-photometer and maximum absorption were observed both crystalline carotin and xanthophyll in CS<sub>2</sub> solution (Mol  $\times 10^{-5}$  conc.) (Fig. 1).

Maximum absorptions were,

Carotin	510~511 $\mu\mu$	476~479 $\mu\mu$	434~435 $\mu\mu$
Xanthophyll	504	473	446

For the determination of carotin in black tea, spectroscopic analysis had been applied in carotin fraction (in pet. ether sol.) which was employed for colourimetric measurement. The standard solution in this case was pure  $\alpha$ -carotin in pet. ether.

Maximum absorptions of carotin fraction in black tea (in pet. ether sol.)

426  $\mu\mu$  448  $\mu\mu$  474  $\mu\mu$   
of standard pure  $\alpha$ -carotin solution (in pet. ether sol.)

425  $\mu\mu$  446  $\mu\mu$  474  $\mu\mu$

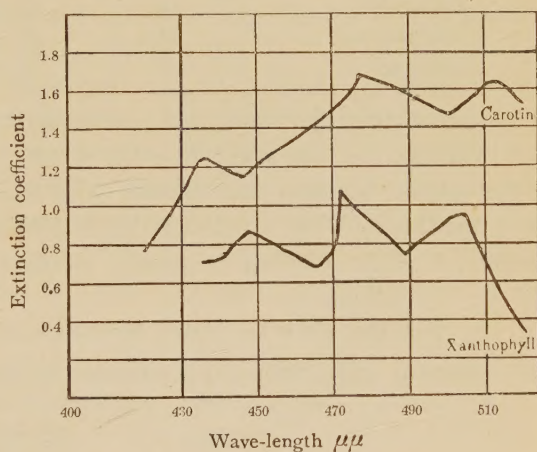


Fig. 1.

*Quantitative analysis of carotinoids.*

In both xanthophyll and carotin 14 g. materials were taken and analyzed according to Willstätter and Stoll<sup>(4)</sup>. For the standard solution 0.0013 g. pure carotin in 100 c.c. pet. ether and 0.0028 g. pure xanthophyll in 100 c.c. ether were employed. The concentration of the standard solution was  $5 \times 10^{-5} \times 1/2$  Mol.

0.1% aqueous potassium bichromate was also used as the standard for xanthophyll.

100 mm. of xanthophyll standard solution  
= 81.0 mm. potassium bichromate solution.

In each solution, colour intensity was compared with standard solution 5 mm. to 45 mm., and mean value was observed, and this converted into 100 mm. The result was,

The reading of colourimeter in mm.

	Carotin		Xanthophyll	
	Water content %	Carotin (standard) 100 mm.	Water content %	K <sub>2</sub> Cr <sub>2</sub> O <sub>7</sub> 100 mm.
Fresh leaf	55	243.6	55	195.1
"	7	110.3	7	97.7
Fermenting tea-leaf	52	261.4	52	208.0
"	12	134.6	12	191.6
Black tea	7	267.6	7	106.3
"	6	307.6	6	132.0
				Standard pure xanthophyll
Oolong tea	6	374.2	6	189.1
Touchang tea	11	127.4	11	106.6
Green tea	5	224.6	6	179.2

Calculation of carotin and xanthophyll gave,

carotin  $50 \times 0.536 \times 1/2 \times 1/2 \times 100/C \times 2.857$  g.

(the original solution was diluted twice its volume in this case)

xanthophyll  $50 \times 0.568 \times 1/2 \times 100/X \times 2.857$  g.

where,  $C$  = the reading of carotin solution equal to 100 mm. standard solution,

$X$  = the same as above for xanthophyll 2.857 = dilution factor.

Carotin and xanthophyll contents in anhydrous 1 kg. material:

	Carotin (g.)		Carotin mean value	Xanthophyll (g.)		Xanthophyll mean value
Fresh leaf	0.1746	0.1757	0.1751	0.4623	0.4465	0.4544
Fermenting tea-leaf	0.1526	0.1617	0.1571	0.4063	0.4411	0.4237
Black tea	0.0769	0.0662	0.0715	0.4103	0.3269	0.3686
Oolong tea	0.7840			0.2411		
Touchang tea	0.1681			0.4257		
Green tea	0.1571			0.4175		

## On the Organic Acids in the Fruits of Ceylon Olive

(*Elaeocarpus serratus* Lin.)

By

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(Received August 16, 1932.)

The fresh fruits and pickles of the fruits of Ceylon olive\* have been eaten by Formosan and south Chinese from long time ago and still in present time.

The fruit has special sour taste and we have investigated its component. The main part of the acids was citric, besides which we have separated a crystalline acid, almost insoluble in water. It crystallized in prisms of mp 215° and it was identified as a mucic acid. Under microscope, we couldn't find any crystal in the fruit, but this crystal was easily observed when the fresh fruit had been boiled in water for twenty minutes and stored in ice-box for two days. These facts suggested that mucic acid must be in free state in the fruit.

Besides acid fractions we observed that the fresh juice indicated strong Benzozonow's Vitamin-C reaction. We have determined its content by the feeding experiment with guinea-pigs. Four cubic centimeters of the juice per day was the minimum quantity to maintain the normal growth of the animal, while in the case of lemon juice 3 c.c. per day was quite sufficient\*.

### Experimental.

To the pulp of fresh fruit, alcohol was added until 70% concentration to precipitate pectinic substance. The alcohol was then evaporated and the residue was neutralized with dilute sodium carbonate solution. The organic acid was then precipitated by lead acetate, and the lead salt was decomposed by sulphuretted hydrogen. The isolation of organic acids was carried out according to Schmidt. Citric acid was thus separated, a trace of tartaric acid was detected, but no oxalic, fumaric, and succinic acid was traced. Besides these acids, crystals of mp 215°, almost insoluble in cold water, were obtained, but the yield was very scanty.

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\* The composition of the pulp of the fruit was:

Water	85.73%	Ash	0.62%
Total acid (as citric acid)	4.37	Crude fiber	1.80
Reducing sugar (as glucose)	1.93	Crude fat	0.23
Non-reducing sugar (as sucrose)	0.98	Nitrogen	0.05

To obtain mucic acid only, alcoholic solution (50%) of mashed pulp was neutralized directly with barium hydroxide. The precipitate was then decomposed by sulphuric acid, and the filtrate was concentrated and cooled. After a while, mucic acid separated out. When recrystallized from boiling water, it forms prismatic crystals of mp 215°. The yield was 0.5% of the fruit.

Analysis of calcium citrate :

Substance (anhydrous)	CaO	Ca %
0.3604 g.	0.1272 g.	24.67
Cal. $(C_6H_5O_7)_2Ca_3$ required		24.13

Analysis of mucic acid :

Substance (mg.)	CO <sub>2</sub> (mg.)	H <sub>2</sub> O (mg.)	C %	H %
9.205	11.480	3.952	34.01	4.80
6.778	8.401	3.020	33.81	4.98
Cal. $C_6H_{10}O_8$ required			34.29	4.76

Silver salt of mucic acid :

Substance (mg.)	Ag (mg.)	Ag %	Mol. weight
22.016	11.161	50.69	
14.675	7.431	50.64	209.88
Cal. $C_6H_5O_8Ag_2$ required		50.91	
Cal. $C_6H_{10}O_8$ "			210.08

Phenylhydrazide from the separated mucic acid melted at 238°, and was identified as pure mucic acid phenylhydrazide.

## On the Red Colouring Matter of *Hibiscus Sabdariffa* L.

(A New Glycoside *Hiviscin*.)

By

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(Received August 16, 1932.)

In 1909 A. G. Perkin<sup>(1)</sup> isolated a yellow colouring matter *gossypetin* from

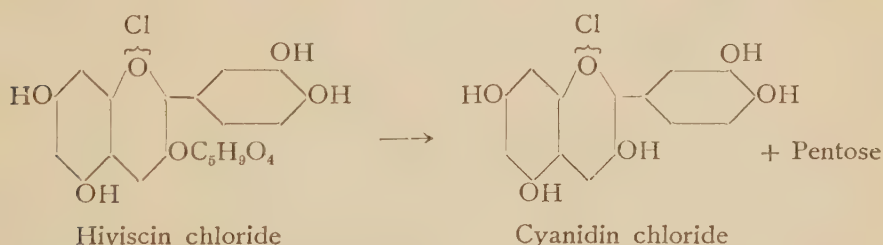
(1) A. G. Perkin: J. Chem. Soc., (1909), 1855.

the calyx of the fruit of *Hibiscus Sabdariffa* L. (Roselle), a small tropical shrub, and this gossypetin had been later synthesized by Baker, Nozu and Robinson<sup>(2)</sup>. The calyx is originally of a deep red colour and is easily suggested that the fruits contain originally red compound besides gossypetin.

The red colouring matter is easily soluble in water, it changes to fine red by hydrochloric acid and by alkali to bluish green, these indicating the presence of anthocyanin. So the investigation of the pigment has been carried out according to Willstätter, Kaller and Robinson.

The dried and powdered material was first extracted with 1.5% methyl alcoholic hydrogen chloride, the pigment was then precipitated by ether, and converted into picrate. When this picrate was decomposed by 7% methyl alcoholic hydrogen chloride, brownish red prisms (m.p. 192°) were obtained, which proved to be an anthocyanin chloride. This red colouring matter is soluble in water, with reddish violet colour, which changes into fine red by hydrochloric acid, into violet blue by sodium carbonate, and very unstable pure blue by sodium hydroxide. The result of oxidation test with ferric chloride showed that it belongs to cyanin group, and moreover, the distribution number between 0.5% hydrochloric acid and amyl alcohol indicated that the pigment is an anthocyanin monoxide<sup>(3)</sup>.

The isolated anthocyanin chloride contained  $4\frac{1}{2}$  mol crystalline water. The analytical result of anhydrous crystal was  $C_{20}H_{19}O_{10}Cl$ , and by hydrolysis it was confirmed that the anthocyanin consists of a cyanidin and one molecule of pentose:—



As the separated anthocyanin was quite different from gossypetin and such an anthocyanin, which is combined with a pentose has not yet been found in nature, so we wish to give the name *hiviscin* to it.

The determination of the nature of the carbohydrate residue could not be made thoroughly on account of scarcity of the materials, but the colour reaction, optical properties and its derivatives confirmed that the carbohydrate must be a pentose.

As for the attaching position of the sugar to cyanidin, we had concerned

(2) Baker, Nozu and Robinson: J. Chem. Soc., (1929), 74.

(3) Robertson and Robinson: Biochem. J., (1931), 1690.

the oxidation test with ferric chloride according to Robinson<sup>(4)</sup>, and the time of decoloration was almost coincided with that of idein and chrysanthemin, indicating that the pentose is probably attached to 3 position of the flavyl group.

The maximum absorption of the spectrum and the colour reaction in buffered solution were also described in the experimental part.

### Experimental

#### *Isolation of hivoscin chloride:*

1.200 kg. dried calyx of the fruits were extracted with 3.6 L. methyl alcoholic hydrogen chloride (1.5%) in room temperature for about 48 hours, filtered on Buchner funnel and again extracted with 2 L. methyl alcoholic hydrogen chloride of the same concentration. After 24 hours, it was filtered and pressed, the total filtrate amounting to 5 L. To this alcoholic extract, 13 L. of ether were added, whereby the colouring matter was deposited as syrupy mass. It was dissolved in 800 c.c. of methyl alcoholic hydrogen chloride (1%) and again precipitated by the addition of 2.5 L. ether. The syrupy colouring matter was now dissolved in 500 c.c. hot water and was converted into picrate by means of 200 c.c. hot saturated aqueous picric acid. The resinous matter was first filtered and the filtrate was cooled in an ice-chest for one week. The crude picrate which crystallized out was collected and the filtrate was again concentrated in vacuum at room temperature (20°) and again cooled in ice-chest for one week, the deposited picrate being rather pure in this case. It was tried to recrystallize the crude picrate from 0.5% methyl alcoholic hydrogen chloride, but the resulted deep red prismatic needles were contaminated with free anthocyanin chloride and the yield was not good. The crude picrate was therefore converted into anthocyanin chloride directly by means of dissolving picrate (3.5 g.) in 2.0% methyl alcoholic hydrogen chloride (200 c.c.) and 800 c.c. of ether was added. After cooling this mixture for 24 hours in an ice-chest, the precipitated colouring matter was collected carefully. This was again dissolved in 0.5% hydrochloric acid (150 c.c.), and the filtrate from resinous matter was added with 7% methyl alcoholic hydrogen chloride (500 c.c.), cooled in ice-chest for 24 hours, when metallic brownish red needle crystal was obtained. After repeating this treatment once more, the anthocyanin chloride was almost pure, but again recrystallized from 1% methyl alcoholic hydrogen chloride m p 192°. The yield was 2 g..

Picrate 0.03 g. anthocyanin chloride was dissolved in 2 c.c. hot water and was added with 1 c.c. hot aqueous picric acid solution. Deep red needles (gathering) of m p 185° (dec.).

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(4) Grove and Robinson: J. Chem. Soc., (1931), 2737.

The anthocyanin chloride thus obtained contains  $4\frac{1}{2}$  mol crystalline water, one mol of which was strongly combined. The anhydrous compound was obtained by drying it at  $100^\circ$  under 1 mm. pressure.

The results of analysis were;

Crystalline water:

0.1938 g. air-dried substance lost (at $100^\circ$ in 1 mm.) water	
in weight 0.0265 g.	H <sub>2</sub> O 15.74%
Cal. for $C_{20}H_{19}O_{10}Cl \cdot 4\frac{1}{2} H_2O$	H <sub>2</sub> O 15.23%

Air-dried substance:

Substance (mg.)	H <sub>2</sub> O (mg.)	CO <sub>2</sub> (mg.)	H%	C %	Cl%
4.090	1.921	6.804	5.26	45.37	
4.925	2.179	8.200	5.19	45.41	
7.143	Rhodan titration 0.275 c.c.				6.51
Cal. for $C_{20}H_{19}O_{10}Cl \cdot 4\frac{1}{2} H_2O$			5.26	45.11	6.56

For the estimation of chlorine Ter-Meulen and Heslingas' method was applied, where 1 c.c. of rhodan solution = 1,900 mg. Cl.

Substance containing 1 mol H<sub>2</sub>O:

0.1032 g. substance (dried at $100^\circ$ in 20 mm.) lost 0.0044 g.	
in weight at $100^\circ$ in 1 mm.	H <sub>2</sub> O 4.26%
Cal. for $C_{20}H_{19}O_{10}Cl \cdot H_2O$	H <sub>2</sub> O 3.82%

Substance (mg.)	H <sub>2</sub> O (mg.)	CO <sub>2</sub> (mg.)	H%	C %	Cl%
2.071	0.826	3.846	4.46	50.65	
3.596	1.446	6.718	4.48	50.96	
6.286	2.470	11.668	4.40	50.63	
3.132	1.238	5.834	4.42	50.80	
6.413	Rhodan titration 0.200 c.c.				7.45
Cal. for $C_{20}H_{19}O_{10} \cdot H_2O$			4.45	50.85	7.51

Anhydrous substance dried at  $100^\circ$  in 1 mm:

Substance (mg.)	H <sub>2</sub> O (mg.)	CO <sub>2</sub> (mg.)	H%	C %	Cl%
2.151	0.835	4.153	4.34	52.66	
3.417	1.432	6.546	4.69	52.25	
7.153	Rhodan titration 0.285 c.c.				7.57
4.742	Rhodan titration 0.185 c.c.				7.41
Cal. for $C_{20}H_{19}O_{10}Cl$			4.18	52.86	7.71

*Properties of hiviscin chloride:*

Hiviscin chloride crystallizes in brownish red long prisms, but under

microscope it seems greyish violet. Its aqueous solution is violet, but the colour changes red by hydrochloric acid. The colouration in aqueous sodium carbonate is violet blue, but on addition of aqueous sodium hydroxide it turns blue, and quickly fades away into greenish yellow. In alcoholic solution hiviscin shows carmine red colour with violet nuance, but on addition of ferric chloride the colour becomes pure blue, which becomes violet when diluted with water. Further, by aqueous lead acetate the colour becomes blue violet and by alum violet, both of them giving stable colouration.

*Oxidation test by ferric chloride:*

Léon and Robinson<sup>(5)</sup> stated, that the time of decolourization of anthocyanin by ferric chloride is different according to the position of carbohydrate residue attached to flavyl group. According to them, for 3 glucoside in cyanin group, the colour is slowly oxidized, and in 1 hour 35 minutes the colour is nearly discharged, while for 5 glucoside (synthetic) colour was destroyed only in 10 minutes.

In the case of hiviscin, 2.020 mg. anthocyanin chloride was dissolved in 50 c.c. 1% hydrochloric acid. and after three minutes 50 c.c. of 0.125% ferric chloride was added. The colouring matter was gradually oxidized, and after 1 hour 40 minutes the colour was almost discharged.

*Distribution number of hiviscin:*

This was carried out by direct comparison method according to Levy and Robinson<sup>(5)</sup>, using 0.5% hydrochloric acid (50 c.c.) and amyl alcohol (50 c.c.). The result was;

Substance (mg.)	in G. mol. $\times 10^{-6}$	Distribution number	
		1st shake	2nd shake
13.00	26.82	15.20	15.38
6.50	13.41	16.61	16.64
3.25	6.69	16.92	17.08
1.30	2.68	17.48	18.12

*Absorption spectrum of hiviscin and cyanidin:*

The maximum absorption in water solution ( $10^{-5}$  mol) was observed using spectrophotometer.

$\mu\mu$	Cyanidin chloride	Hiviscin chloride	$\mu\mu$	Cyanidin chloride	Hiviscin chloride
600	0.29	0.38	555	0.46	0.60
590	0.33	0.43	550	0.42	0.59
580	0.35	0.59	545	0.41	0.58
575	0.38	0.62	540	0.40	0.58
570	0.43	0.62	530	0.38	0.54
565	0.46	0.61	520	0.36	0.46
560	0.48	0.61			

(5) Levy and Robinson: J. Chem. Soc., (1931), 2720.

(5) loc. cit.

Maximum absorption lies on  $560\ \mu\mu$  and  $570\sim 575\ \mu\mu$  with cyanidin chloride and hiviscin chloride respectively.

*Colour reaction in buffered solution:*

The method of Robertson and Robinson<sup>(6)</sup> was employed and the numbers refer to solution of PH 3.2 (1) to PH 11.0 (14) and then to more alkaline solution.

1% and 20% hiviscin hydrochloric acid, stable eocin red.

(1) light pink, stable.

(2) almost the same as (1), but with slight violet nuance, after ten minutes, the colour became light red violet,

(3) violet pink.

(4) the same as (3).

(5) reddish violet.

From (3) to (5) fading into light violet pink after 30 minutes.

(6) reddish violet, deeper in violet than (5).

(7) the same as (6).

(8) light red violet.

These three fading to light red violet after 30 minutes.

(9) light red violet, but lighter than (8).

(10) light violet red.

(11) the same as (10).

(12) light violet.

These four fading more rapidly, after 30 minutes very light red violet.

(13) violet, faded very rapidly.

(14) the same as (13), faded very rapidly.

(15) bluish violet, faded very rapidly.

(16) light blue, faded very rapidly.

(17) light blue, faded very rapidly.

*Hydrolysis of hiviscin chloride:*

(1) 0.1606 g. anhydrous material was dissolved in 6 c.c. hot water, added with 6 c.c. concentrated hydrochloric acid and was boiled 2 minutes. After cooling the cyanidin chloride was filtered. It crystallized in deep red violet gathering needles: yield 0.0924 g. (anhydrous).

(II) 0.1430 g. anhydrous material was dissolved in 3 c.c. hot water, added with 3 c.c. concentrated hydrochloric acid and was boiled 2 minutes: yield of anhydrous cyanidin chloride was 0.0987 g.

*Analysis of cyanidin chloride:*

The material dried at  $100^{\circ}$  in 1 mm. pressure was analyzed.

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(6) Robertson and Robinson: Biochem. J., (1929), 35.

Substance (mg.)	H <sub>2</sub> O (mg.)	CO <sub>2</sub> (mg.)	H%	C%	Cl%
7.951	2.704	16.132	3.81	55.33	
3.313	1.008	6.750	3.39	55.57	
9.448	Rhodan titration 0.590 c.c.				11.27
Cal. for C <sub>15</sub> H <sub>11</sub> O <sub>6</sub> Cl			3.41	55.80	11.00

*Properties of cyanidin chloride:*

In amyl alcohol solution the colour is red violet which turns violet by aqueous sodium acetate and blue by ferric chloride. In alcoholic ferric chloride solution the colour is blue and changes into greenish yellow when it is diluted.

Oxidation test by aqueous ferric chloride was carried out in the same way as with hiviscin chloride. After three minutes fading of the colour occurred, and ten minutes later the colour was nearly oxidized and after in thirty minutes it was almost decolourized. The colour reaction in buffered solution showed the same colouration as is given by Robertson and Robinson<sup>(7)</sup> in the case of cyanidin chloride.

*Analysis of carbohydrate:*

The filtrate from the hydrolysis of hiviscin chloride was neutralized with sodium carbonate and was freed from the remaining colouring matter with amyl alcohol. The aqueous solution was then concentrated, added with alcohol to precipitate mineral matter, the alcoholic solution was evaporated, decolourized by animal charcoal and filled up 20 c.c. The sugar content was estimated by volumetric method.

- I. 0.1066 g. anhydrous hiviscin chloride gave 0.042 g. xylose or 0.0392 g. arabinose.
- II. 0.1430 g. anhydrous hiviscin chloride gave 0.043 g. xylose or 0.041 g. arabinose.

Since, in the cause of hydrolysis of hiviscin, evolution of furfural was observed, its content was estimated as phloroglucid. 23.307 mg. hiviscin chloride gave 4.974 mg. furfural phloroglucid corresponding to 7.746 mg. pentose (33.23 %) where C<sub>20</sub>H<sub>16</sub>O<sub>10</sub>Cl required 33.24% pentose. The maximum absorption of the above phloroglucid lies on 580  $\mu\mu$ . This excludes the existence of methyl furfural phloroglucid, whose absorptions lie below 480.

*The colour reaction of the carbohydrate:*

(1) Naphthoresorcin test: green fluorescence, absorption on 520  $\mu\mu$ , the colour was insoluble in ether. (2) Phloroglucin test: red. (3) Bial's test: positive (green). (4) Thomas' reaction: positive. (5) Pinoffs' test: positive, after a minute violet red.

(7) loc. cit., 37.

*Derivatives:*

Phenyl osazone was prepared in ordinary way. Recrystallized from 10 % alcoholic solution, m p  $192^{\circ}$  (yellow needle), *p*-brom-*o*-phenylosazone was also prepared. To 0.3 g. *p*-brom-*o*-phenylhydrazine in 3 c.c. hot water was added 0.1 g. sugar dissolved in 0.5 c.c. water and 1 c.c. of 50 % aqueous acetic acid, and the mixture was heated on water-bath for 10 minutes. On cooling, the crystals separated out which recrystallized from 50 % alcoholic solution form yellow needles of m p  $162^{\circ}$ .

*The aqueous sugar solution is dextrorotatory:*

These results indicate that the carbohydrate is a pentose and attached to cyanidin in molecular proportion, but further investigation is required in these respects.

## The Catechin in the Fruit of *Areca catechu* L.

By

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(Agricultural Chemical Department, Taihoku Imperial University, Taiwan, Japan.)

(Received August 16, 1932.)

According to A. G. Perkin<sup>(1)</sup> areca catechin seems to resemble acacia catechin, but its pure isolation has not yet been succeeded. The fresh pulp of the fruit is colourless and soluble in water, but when it is dried in air it becomes insoluble brown matter, having an appearance of a phlobaphene. The fresh pulp must, therefore, be taken for a starting material.

The attempt of the isolation of catechin from the fresh fruit was successful. The crystal we had obtained was colourless needles m p  $96^{\circ}$  (the anhydrous form m p  $175^{\circ}$ ). The analytical results agreed with  $C_{15}H_{14}O_6$ , and its optical properties and its penta-acetyl derivative  $[C_{15}H_9O(OCOCH_3)_5]$  identity with *d*-catechin which we had isolated from gambier catechu<sup>(2)(3)</sup>.

### Experimental.

The mashed pulp from 800 g. fresh fruit was extracted twice with each

(1) A. G. Perkin: The Natural Organic Colouring Matters, 463.

(2) A. G. Perkin and E. Yoshitake: J. Chem. Soc., **81** (1902), 1160.

(3) Freudenberg and Purrmann: Ann. Chem., **437** (1924), 274

1 l. alcohol (94%) for two hours at 80°. The united filtrate was evaporated in vacuum in the atmosphere of carbon dioxide, and the catechin was precipitated as lead salt, the latter having decomposed by sulphuretted hydrogen. The filtrate from lead sulphide was concentrated in vacuum to a syrup, which was then extracted with ether using a separating funnel. The ethereal solution was then concentrated and extracted with a little water. When this water solution was cooled, needle crystals separated, melting at 96°. The crystals contain 4 mol crystalline water to be dehydrated in vacuum at 100°. The anhydrous substance melted at 175°. Yield was 3 g.

With ferric chloride it gives a green colour, with bromine water a yellowish and with lime water a brownish red precipitate. It is precipitated when kept in contact with air, and other properties showed perfectly that it is identical with *d*-catechin from gambier.

Crystalline water :

1.0036 g. air-dried material lost at 100° in vacuum 0.2060 g.

in weight

H<sub>2</sub>O 20.52%

Cal. C<sub>15</sub>H<sub>14</sub>O<sub>6</sub>•4H<sub>2</sub>O

H<sub>2</sub>O 19.89%

Analytical results :

Substance (mg.)	H <sub>2</sub> O (mg.)	CO <sub>2</sub> (mg.)	H %	C %
5.219	2.014	11.887	4.32	62.13
2.982	1.078	6.836	4.42	62.53
C <sub>15</sub> H <sub>14</sub> O <sub>6</sub> requires			4.82	62.07

The rotation of *d*-catechin from *areca catechu* :

0.3125 g. was dissolved in 25 c.c. water containing a little acetone.

$$[\alpha]_D^{19} = (+ 0.20^\circ \times 25) : (1 \times 1.25) = +16.0^\circ$$

The absorption bands of spectrum were just the same as that of *d*-catechin from gambier.

*Acetyl derivative :*

0.5 g. dried material was dissolved in 7 c.c. well cooled pyridine, acetylated by 7 c.c. of acetylchloride. The mixture was then poured into ice-water and the precipitate was crystallized from alcohol acetone mixture ; colourless needles, m p 128°, yield 0.6 g.

The analytical results :

The material dried at 100° in vacuum and analyzed.

Substance (mg.)	H <sub>2</sub> O (mg.)	CO <sub>2</sub> (mg.)	H %	C %
5.458	2.357	12.001	4.79	59.96
3.119	1.349	6.858	4.80	59.96
C <sub>15</sub> H <sub>9</sub> O(OCOCH <sub>3</sub> ) <sub>5</sub> requires			4.80	60.00

The rotation of penta-acetyl *d*-catechin from *areca catechu* :

0.4446 g. dried substance was dissolved in 25 c.c. acetylene tetrachloride.

$$[\alpha]_D^{19} = (+ 0.74^\circ \times 25) : (1 \times 1.7784) = + 41.6^\circ.$$

## On the Chemical Composition, especially Organic Bases of "Di-Saké".

By

Kotaro NISHIDA.

(Kagoshima Agricultural College, Kagoshima, Japan.)

(Received September 12, 1932.)

The "di-saké" or "di-shu" is a kind of "saké", which is alcoholic beverage peculiar to the Japanese ; and brewed in only two prefectures—Kagoshima and Miyazaki—in Japan. The annual production of "di-saké" in these districts amounts to about 3,400~3,600 *koku* or 6,200~6,500 hectolitres in round number. Its raw-materials, the same with "saké" : that is (1) steamed rice, (2) "koji", the fungoid mass of *Aspergillus Oryzae* grown on steamed rice, and (3) Water ; but this steamed rice as raw-material of "di-saké" was made by the very roughly polished rice than that of the "saké", and then the method of brewing differ from "saké".

In the case of brewing of "di-saké", using in comparatively small amount of water and large amount of aged "koji", the saccharification of mash is promoted and the fermentation controlled ; and to neutralize the acids in fermented mash, wood ash is added into the mash before the press ; and then new "di-saké" obtained by the press is not pasteurized.

The "di-saké" is a sweet liquor of a yellow or yellowish brown colour and of the consistency more viscous than that of ordinal "saké" ; and its use is not only for drinking, but for cooking instead of "mirin".

### Experimental Results.

The "di-saké" experimented with was brewed at Kagoshima City ; and the analytical results of the sample are shown in the following table ;

	g. in 100 c.c.	g. in 100 g.
Crude protein	2.413	2.259
Protein	0.056	0.052
Extract	20.224	18.934
Reducing Sugar (as glucose)	12.880	12.059
Dextrin	1.652	1.547
Ash	0.260	0.243
Specific gravity		1.0681
Alcohol	12.89	
" vol. %		16.24
pH		6.823

The various forms of nitrogen in the sample were also determined:

	g. in 100 c.c.	Ratio (Total N as 100)
Total N	0.386	100.0
Protein N	0.009	2.3
Non-protein N	0.377	97.7
In which Ammonia N	0.029	7.5
Organic base N	0.074	19.2
Other N	0.274	71.0

### Isolation and Identification of Organic Bases.

For the isolation of the organic bases 10 litres of the "di-saké" were evaporated under reduced pressure to about 4 litres. After this operation the protein substance and other impurities were removed by neutral- and basic-lead acetate, and excess of the lead by  $H_2SO_4$ , and then the organic bases were precipitated by phosphotungstic acid. And according to the general method the precipitate formed by phosphotungstic acid was fractionated into three fractions, and researches were made about the organic bases in each fraction.

#### (1) Purine Base-Fraction ( $AgNO_3$ -precipitate):—

The yield of the base from this fraction was 0.20 g. as hydrochloride. Its picrate formed yellow prisms, changed to black colour at about  $210^\circ C$  (uncorr.). On analysing it as its chloroplatinate, following result was obtained:

0.0752 g. Subst.	0.0217 g. Pt.	28.86% Pt.
Calc. for Hypoxanthine-chloroplatinate $[(C_5H_4N_4O \cdot HCl)_2PtCl_4]$		28.62% Pt.

The chloroaurate of the base formed yellow prisms, decomposed at  $254^\circ C$  (uncorr.), and the result of the analysis was as follows:

0.1647 g. Subst.	0.0682 g. Au.	41.41% Au
Calc. for Hypoxanthine-chloroaurat ( $C_5H_4N_4O \cdot HCl \cdot AuCl_3$ )		41.42% "

These results in all respects agree precisely with hypoxanthine derivatives.

(2) Arginine-Fraction ( $AgNO_3$  &  $Ba(OH)_2$ -precipitate) :—

The yield of the base from this fraction was 4.60 g. as nitrate. The nitrate forms chalky substance, and the result of the analysis by Nitron method was as follows :

0.2049 g. Subst.	0.3193 g. $C_{20}H_{16}N_4 \cdot HNO_3$	
	0.05364 g. $HNO_3$	26.18% $HNO_3$
Calc. for Arginine-nitrate ( $C_6H_{11}N_4O_2 \cdot HNO_3$ )		26.55% $HNO_3$

The coppennitrate formed deep blue needles, melted at  $112^\circ C$  (uncorr.) and decomposed at  $230^\circ C$  (uncorr.).

0.1226 g. Subst.	0.0179 g. CuO	0.0143 g. Cu	11.67% Cu
Calc. for Arginine-copper-nitrate [ $(C_6H_{11}N_4O_2) \cdot Cu(NO_3)_2$ ]			11.86% Cu

According to the above results this base seems to coincide with arginine.

(3) Lysine-Fraction (Filtrate from  $AgNO_3$  &  $Ba(OH)_2$ -precipitate) :—

The hydrochloride, which was freed from water obtained by this fraction, was treated with cold absolute alcohol and separated into two portions.

(a) Insoluble portion by cold absolute alcohol :—

Yield ; 0.50 g. This chloride was identified as KCl.

(b) Dissolved portion by cold absolute alcohol :—

Saturated alcoholic solution of  $HgCl_2$  was added to this portion.

The hydrochloride of the base obtained from the  $HgCl_2$ -precipitate, forms colorless, very hygroscopic, large prisms and gives the alloxan reaction. The chloroaurate prepared from the hydrochloride forms yellow mossy crystals, and is sparingly soluble in water ; the melting point was determined as  $256-257^\circ C$  (uncorr.) ; and the analysis gave the following results :

0.1541 g. Subst.	0.0689 g. Au	44.71% Au
0.2605 g. "	0.1157 g. "	44.41% "
Calc. for cholinechloroaurate ( $C_5H_{14}NOCl \cdot AuCl_3$ )		44.49% "

The chloroplatinate formed orange yellow prisms, easily soluble in water, melted at  $236-237^\circ C$  (uncorr.) with decomposition, and gave the following analysis :

0.2345 g. Subst.	0.0734 g. Pt	31.30% Pt
0.3581 g. "	0.1118 g. "	31.22% "
Calc. for cholinechloroplatinate [ $(C_5H_{14}NOCl)_2PtCl_4$ ]		31.64% Pt

The above results agree fairly with choline derivatives ; the yield of the base was 6.75 g. as chloroaurate.

### Summary.

The chemical composition of the "Di-saké" compared with that of the ordinal "saké" is summarized as follows :

(1) In the above experimental results the nitrogenous compounds isolated from 10 litres of "di-saké", are hypoxanthine-hydrochloride (0.20 g.), arginine nitrate (4.60 g.), cholinechloroaurate (6.75 g.), and ammonia (3.52 g.), while the isolated bases from 25 litres of "saké" by Dr. K. Kurono, are cholinepicrate (3.8 g.), histaminepicrate (0.15 g.), lysinepicrate (0.52 g.) and ammonia (1.3 g.). It is the most great difference that the "di-saké" contains large amount of arginine, which could not be isolated from the "saké".

(2) As regards the general composition, 1 find great difference between the "di-saké" and the "saké": in the former, the quantity of reducing sugar (chiefly glucose) and other extractive substances are considerably much more than those of the latter.

(3) In the "di-saké", the amount of non-protein substances is much more than those of the "saké".

(4) The reaction of "di-saké" is almost neutral, but in the "saké" remarkably acidic.

## Researches on the Electrolytic Reduction Potentials of Organic Compounds, Part XVI.

Reduction potential of *p*-aminoazobenzene.

By

Masuzo SHIKATA and Isamu TACHI.

(Received September 19, 1932.)

### Summary.

The results of the investigation on the electrolytic reduction potential of *p*-aminoazobenzene with the polarograph and dropping mercury cathode at 25°C were as follows.

(1) Reduction potential of *p*-aminoazobenzene was more positive in lower concentration than that in higher concentration in every PH solution.

(2) Owing to the weak basic property of *p*-aminoazobenzene, there were two reduction potentials which were due to the dissociated and undissociated forms in some proper acidic solutions. In high acidic solution, *p*-aminoazobenzene changed to quinoid form which was demonstrated by spectrographic study. The reduction potential of quinoid form was more positive than that of azoid form and further, that of dissociated form more posi-

tive than that of undissociated form.

(3) The reduction potential of *p*-aminoazobenzene which substituted  $\text{NH}_2$  group in azobenzene was more negative than that of the latter. This is expected from our negativity rule of electrolytic reduction.

(4) The solubility of *p*-aminoazobenzene in water at  $25^\circ\text{C}$  was found to be  $2.815 \cdot 10^{-4}$  g. mol per litre calculated by the result of polarographic measurement.

## Researches on the Electrolytic Reduction Potentials of Organic Compounds, Part XVII.

Reduction potential of dimethylaminoazobenzene.

By

Isamu TACHI.

(Received September 19, 1932.)

### Summary.

The results of the investigation on the electrolytic reduction potential of dimethylaminoazobenzene with the polarograph and dropping cathode were as follows:

(1) The reduction potential of dimethylaminoazobenzene was more negative in high concentration than that of in low concentration, except in the case of solution which  $P_H$  was lower than 2.2.

(2) In acidic solution, the reduction of dimethylaminoazobenzene showed the reduction potentials of quinoid, dissociated and undissociated forms of azoid form as same as in the case of *p*-aminoazobenzene.

(3) The maximum currents of the polarograms of dimethylaminoazobenzene in acidic solutions were increased with elevation of concentration of ethanol. From these facts, we assume that dimethylaminoazobenzene becomes easily adsorbable on the mercury cathode in acidic solutions.

(4) The mutual relation among the reduction potentials of azobenzene, *p*-aminoazobenzene and dimethylaminoazobenzene was found to follow our negativity rule of electrolytic reduction, that is, reduction potential of azobenzene was most positive and that of dimethylaminoazobenzene most negative among them.

## Ueber die Verdauungsenzyme der Seidenraupen (*Bombyx Mori*, L.)

Von

Kazuo YAMAFUJI.

(Aus dem Biochem. Laboratorium der Univ. Kyushu, Fukuoka, Japan)

(Eingegangen am Oktober 9, 1932.)

### I. Einleitung.

Als Verdauungsenzyme der Seidenraupen haben mehrere Forscher Amylase, eine trypsinähnliche Protease und Lipase in den Säften der Digestionskanäle, aber Invertase als Endoenzym in den Wänden derselben festgestellt.

Zur rationellen Züchtung der Seidenraupen, ist es nötig, die Natur dieser Enzyme gründlich zu berücksichtigen. Zur Untersuchungen der Erbllichkeit der chemischen Eigentümlichkeiten in den Lebewesen, hat der Verfasser die Wirkungskräfte dieser Enzyme im Körper der Raupen beobachtet, und zwar zuerst inbezug auf die Invertase, deren Enzymnatur nicht genügend bekannt ist, er erforschte den Einfluss der Wasserstoffionenkonzentration und Temperatur auf die Enzymwirkung, und auch bezüglich der Amylase und Protease bestimmte ihre Wirkungskraft in einigen Originalrassen der Seidenraupen.

### II. Einfluss der $P_H$ und Temperatur auf die Invertasewirkung.

1) Darstellung der Enzymlösung:- Die aus den Seidenraupen herausgenommene Wände der Digestionskanäle wurden zur Entfernung des Saftes, des Blutes und anderer Substanzen, zunächst mit 0.85 proz. Kochsalzlösung und dann mit Wasser genügend gewaschen. Diese Wände wurden in einer Reibschale mit feinkörnigem Quarzsand gut verrieben; das Gemenge mit wasserhaltigem Glycerin verrührt, nach eintägigem Stehen bei Zimmertemperatur zentrifugiert und die überstehende Flüssigkeit filtriert. Die so erhaltene Enzymlösung ist durchsichtig.

2) Bestimmungsmethode der Enzymwirkung:- Ich verwandte Sörensen'sche Zitrat-, Phosphat- und Glykokoll-gemische als Pufferlösungen. Die Saccharoselösungen, unter verschiedenen Pufferzusätzen, wurden mit Enzymlösungen gemischt, nach verschiedener Versuchsdauer wurden 2 *N* Sodalösungen zugesetzt und die Drehungsabnahme gemessen.

3) Einfluss der  $P_H$ :- Ich will im folgenden ein Beispiel der Experimente kurz beschreiben. 4 ccm, 30% iger Saccharoselösung wurden mit 4 ccm. Enzymlösung, 8 ccm. Pufferlösung und Toluol versetzt. Nach 18 Stun-

den bei 37°C wurden 10 ccm. dieser Flüssigkeit in 2 ccm. 2 *N* Sodalösung zugesetzt und der Drehungsgrad beobachtet.

$P_H$	4.64	5.01	5.75	6.23	6.59	7.06
Drehungsabnahme (°)	0.06	0.35	0.99	0.80	0.80	1.04
Saccharosespaltung (%)	1.27	7.42	20.97	19.07	19.07	22.03
$P_H$	7.53	7.78	8.34	8.68	9.45	9.82
Drehungsabnahme (°)	0.84	0.85	0.94	1.10	1.12	0.78
Saccharosespaltung (%)	17.80	18.01	19.92	23.31	23.73	16.53

Die Ergebnisse des oben erwähnten und anderer Versuche, welche im Jour. Agr. Chem. Soc. Japan (Japanisch) dargestellt worden sind, weisen darauf hin, dass die saccharosespaltende Kraft dieses Enzyms sehr schwach ist, unter  $P_H = 4.0$  hat es fast keine Wirkung, aber bei alkalischer Einwirkung ist sie etwas höher und zwischen  $P_H = 5.5$  und 10.0 besteht nahezu die gleiche Wirkungskraft und bei  $P_H = 9.5$  liegt das Wirksamkeitsoptimum.

Diese Resultate interessieren im Vergleich zu der Tatsache dass, die Wasserstoffionenkonzentration des Digestionssaftes und des Blutes der Seidenraupen nahe  $P_H = 6.5$  und  $P_H = 10.0$  liegt; bei  $P_H 9.0 \sim 10.8$  liegt die optimale Wirkungen der Amylase und Protease des Digestionssaftes.

4) Einfluss der Temperatur:— Zu 5 ccm. 30%iger Saccharoselösung wurden 15 ccm. Glykokoll-Puffergemisch von  $P_H = 9.5$  addiert. Diese Flüssigkeiten und 10 ccm. Enzymlösungen wurden auf 20 Min. bei verschiedenen Temperaturen gehalten, gemischt und nach 20 Min. bzw. 40 Min. je 10 ccm. dieser Lösungen mit 2 ccm. 2 *N* Sodalösung versetzt. Die unter diesen gleichartigen Bedingungen durchgeführten Untersuchungen zeitigten die nachstehenden Ergebnisse:

Nach 40 Min.

Temperatur (°C)	10.0	25.0	31.0	37.0	45.0	55.6	70.0
Drehungsabnahme (°)	0.32	0.36	0.39	0.41	0.45	0.25	0.15
Saccharosespaltung (%)	9.30	10.47	11.34	11.92	13.08	7.56	4.36

Die Invertasewirkung ist also selbst bei 10°C beträchtlich, wird mit steigender Temperatur stärker und erreicht das Optimum bei 45°C, wird dann aber nach 40 Min. bei 70°C zerstört.

### III. Beziehungen zwischen den Rassen der Seidenraupen und den Wirkungskräften der Amylase und Protease der Digestionssäfte.

1) Rasse und Züchtung:— Ich Wählte Japan-Gynryu, Japan-110, China-7-B, China-7-D, Europa-7-A und Europa-7-C als Originalrassen; China ×

Europa-E und China  $\times$  Europa-F als Bastardrassen aus. Diese 8 Rassen wurden unter gleichen Bedingungen zu derselben Jahreszeit aufgezogen.

2) Gewinnung des Digestionssaftes:- Wenn die Seidenraupen Chloroform riechen, brechen sie die Verdauungssäfte aus. Diese Säfte wurden in Gefäße mit Toluol gesammelt und sofort gebraucht.

3) Bestimmungsmethode der Enzymwirkung:-

a) Dextrinbildung aus Stärke:- Die 1%igen Stärkelösungen wurden mit den gleichen Volumina M/10 Glykokoll-Puffergemisch von  $P_H = 9.08$  vermischt. Als Enzymlösung wurde stets der mit 0.85%iger Kochsalzlösung verdoppelte Verdauungssaft benutzt und im wesentlichen nach dem Verfahren von Wolgemuth die Enzymwirkung bestimmt.

b) Verzuckerung der Stärke:- 15 ccm. 5%iger Stärkelösung wurden mit 15 ccm. M/10 Glykokoll-Puffergemisch von  $P_H = 8.67$  vermenget, diese Lösung, nach 20 Min. bei 30°C, durch den mit 0.85%iger Kochsalzlösung verdoppelten Digestionssaft auf eine Stunde bei 30°C stehen gelassen und der entstandene Zucker mittels der Methode von Bertrand bestimmt.

c) Eiweisspaltung:- Die in M/10 Glykokoll-Pufferlösung von  $P_H = 10.74$  gelöste 0.25%ige Kaseinlösung wurde durch Enzymlösung, welche mit 0.85%iger Chlornatriumlösung hundertfältig verdünnt wurde, eine Stunde bei 30°C stehen gelassen, das ungespaltene Substrat mit 1.5 %iger Trichlor-essigsäure gefällt und nephelometrisch bestimmt.

4) Ergebnisse der Versuche:- Verf. gibt einige seiner Versuchsergebnisse in der Tabelle wieder. Die Zahlen in dieser Tabelle erweisen: Dextrinbildung — ccm. 1 proz. Stärkelösung, welche durch 1 ccm. Digestionssaft in einer Stunde bei 30°C und  $P_H = 9.08$  in Dextrin gespalten wurden; Verzuckerung — ccm. 0.5 %iger Kaliumpermanganatlösung, welche dem durch 1 ccm. Digestionssaft während einer Stunde bei 30°C und  $P_H = 8.67$  entstandenen, aus Stärke invertierten Zucker entsprechen; Eiweisspaltung — mg. Kasein, welche durch 1 ccm. des mit 0.85 %iger Kochsalzlösung hundertfältig verdünnten Digestionssaftes während einer Stunde bei 30°C und  $P_H = 10.74$  gespalten wurden.

	Japan-Ginryu	Japan-110	China-7-B	China-7-D	Europa-7-A	Europa-7-C	China-Europa-E	China-Europa-F
Dextrinbildung	7.5	10.0	10.0	30.0	0.0	0.0	10.0	7.5
Verzuckerung	5.58	8.68	8.68	18.60	1.86	0.62	4.96	4.96
Eiweisspaltung	33.34	33.50	34.82	31.90	33.34	32.41	32.09	32.81

Die stärkespaltende Kraft, sowohl die Dextrinbildung, als auch die Verzuckerung, ist am stärksten bei China-7-D; danach bei China-7-B und Japan-110; und darauffolgen Japan-Ginryu, China  $\times$  Europa-E und China  $\times$  Europa-F

F; bei Europa-7-A und Europa-7-C aber ist sie sehr schwach. Die anderen experimentelle Data, die in der Originalarbeit beschrieben wurden, zeigen dass diese Kraft bei gleichem Alter von Tag zu Tag stärker wird. In der Eiweisspaltbarkeit des Digestionssaftes, unter den oben erwähnten experimentellen Bedingungen, besteht ein Unterschied in den Rassen der Seidenraupen nicht.

### Zusammenfassung.

1) Die Invertase, welche in den Wänden des Digestionskanals der Seidenraupen vorkommt, wirkt zwischen  $P_H = 5.5$  und  $= 10.0$  ungefähr gleich stark und zwar liegt das Optimum etwa bei  $P_H = 9.5$

2) Die saccharosespaltende Kraft dieser Invertases wird mit steigender Temperatur stärker; das Maximum liegt bei  $45^\circ\text{C}$ . Bei  $70^\circ\text{C}$  wird sie innerhalb 1 Stunde zerstört.

3) Unter 8 Rassen der Seidenraupen, besteht die folgende Reihenfolge für die Wirksamkeit der in den Digestionssäften vorkommenden Amylase gefunden: China-7-D > China-7-B > Japan-110 > Japan-Ginryu, China  $\times$  Europa-E, China  $\times$  Europa-F > Europa-7-A, Europa-7-C.

4) Es besteht kein Unterschied in der Kaseinspaltbarkeit der Verdauungssäfte der verschiedenen Rassen.

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## A Study on the Effects of Fatty Acid on Nutrition

By

UME TANGE.

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Until recently, it has been considered that the essential function of fats in the body is to act merely as fuel to the tissues and that when the supply of fat-soluble and other vitamins are sufficient fats can be omitted from the dietary<sup>(1)(2)(3)</sup>.

However, since in 1928, Evans and Burr<sup>(4)</sup> proved definite subnormal

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(1) Hinhede: Chem. Zentr., **2** (1918), 745.

(2) T. B. Osborne and L. B. Mendel: J. Biol. Chem., **45** (1920—1921), 145.

(3) J. C. Drummond and K. H. Coward: Lancet, **2** (1921), 698.

(4) H. M. Evans and G. O. Burr: Proc. Soc. Exp. Biol. and Med., **25** (1928), 390.

growth and irregular ovulation on diet which was complete in every respect except for fats, a considerable emphasis has been put on the fat requirement of the animal. The next year, Burr and Berr<sup>(5)</sup> noted a new dietary deficiency in fat-free diet, by which rats developed a characteristic symptom, the so-called scaly tail condition. About the same time, McAmis, Anderson, and Mendel<sup>(6)</sup> reported subnormal weight of animals on a low fat diet, thus supporting the view that fats are beneficial to the rats. Furthermore, Burr and Burr<sup>(7)</sup> indicated that linoleic acid was highly important and the rats receiving the acid lost the scales and dandruff from their feet and back and were cured. This investigation called the author's attention to determine what kinds of fatty acids and their glycerides are essential and what rôle they play in nutrition.

The present paper is concerned with some dietary deficiency developed in the rigidly fat-free diet and with the rôle of unsaturated fatty acids.

#### *Preparation of Basal Diet and of Animals.*

Casein:- 110 g. of high grade casein were washed with distilled water twice daily for 2 days. After draining the washed water, the casein was transferred into 1100 c.c. of 0.09 normal NaOH with a little amount of toluene and shaken for about 20 hours. The alkaline solution was then treated with a 1:1 mixture of normal hydrochloric acid and acetic acid under violent stirring. The precipitated casein was washed with distilled water until the chloride test was almost negative, and the water was pressed out by suction. The casein was now put into 95 per cent alcohol to remove the moisture. After pressing out the alcohol as completely as possible, the casein was spread on the filter paper to dry at ordinary temperature; the dry powdered casein was extracted with ether for 7 days. The prolonged extraction was very important in producing a casein free from fat.

Starch:- Potato-starch (Japanese pharmacopoeia) was boiled with 95 per cent alcohol containing hydrogen chloride gas according to the direction given by Taylor and Nelson<sup>(8)</sup>. The purified starch obtained in this way was extracted with ether for 7 days. Later, the procedure was modified by mere extraction with 95 per cent alcohol, as there were no different effects upon the rats. Taylor and Nelson have shown that the major parts of the fatty substance present in starch cannot be removed by solvent without previous hydrolysis. They have also indicated that in the case of potato-starch there is only a trace of the "fat by hydrolysis", which is almost negligible. Moreover, recent investigation of Evans and Lepkovsky<sup>(9)</sup> has proved that potato-

(5) G. O. Burr and M. M. Burr: J. Biol. Chem., **82** (1929), 345.

(6) A. J. McAmis, W. E. Anderson and L. B. Mendel: J. Biol. Chem., **82** (1929), 247.

(7) G. O. Burr and M. M. Burr: J. Biol. Chem., **86** (1930), 587.

(8) T. C. Taylor and J. M. Nelson: J. Am. Chem. Soc., **42** (1920), 1726.

(9) H. M. Evans and S. Lepkovsky: J. Biol. Chem., **96** (1932), 143.

starch was ineffective as a cure of the disease that resulted from fat-free diet. Yeast extract:- 400 g. of dry powdered yeast (baker's yeast prepared by Oriental Yeast Company in Tokyo) were added to 1600 c.c. of 50 per cent ethylalcohol and stirred continuously for 1/2 hour at room temperature; then the mixture was filtered through muslin in a fruit-press or a Buchner funnel. The residue was treated in like manner with 65 per cent alcohol and filtered as before. The residue, this time, was transferred into 75 per cent boiling alcohol of the same volume as above, the boiling was continued for one hour, let settled for some hours, and then filtered. The treatment was repeated once more, but with 85 per cent alcohol.

All the filtrates were combined and evaporated under reduced pressure. The concentrated extract was then slightly acidified with hydrochloric acid and shaken with ether to remove fat completely; the ether was evaporated off. The solution was diluted with distilled water to a volume of 400 c.c. and preserved in the ice-box. This extract should have contained all of the water-alcohol-soluble vitamin B complex of yeast. Each c.c. was equivalent to 1 g of the original yeast.

Basal diet:- The diet consisted of 21% purified casein, 75% purified starch and 4% McCollum salt mixture, supplemented with 5 c.c. of the yeast extract for every 100 g of the diet. As vitamin A and D sources, one drop of 1% biosterol\* free from fat and of 0.01% irradiated ergosterol† were given per rat per day respectively, both dissolved in liquid paraffin. Later, 0.1% ergosterol solution was used since 0.01% solution might be insufficient to prevent rickets. Iodine water was furnished once a week.

Animals:- In all the experiments male albino rats of 35-40 g. of weight were used, and 2 or 3 of them were kept in a cage with the raised bottom of coarse wire-screen to prevent accessibility to feces. The solid fatty acids were simply mixed with the basal diet while the liquid fatty acids were given by means of a dropping pipette. Positive results were marked by the recovery of symptoms and renewed growth. In our laboratory the diet is usually cooked in a semisolid state to prevent spilling.

#### *Fatty Acids Used and Their Preparation.*

The fatty acids\*\* used in this experiment are listed as Table I<sup>(10)</sup>.

Stearic acid was recrystallized from alcohol.

Clupanodonic acid was prepared from methyl ester of the acid according to the direction of Sahashi: 40 g of the methyl ester were dissolved in 400

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\*† I wish to thank Dr. M. Sumi and Mr. S. Hamano for supplying the irradiated ergosterol and biosterol.

\*\* I desire to thank Dr. Y. Sahashi for generously supplying the pure fatty acids.

(10) Y. Sahashi: "Riken Iho", 11 (1932), 1075.

cc of 5% alcoholic NaOH and left overnight at room temperature, then the solution was diluted with distilled water twice the volume. The solution

Table I.

	b p/m p	d	$n_D$	Acid value	Iodine value
Oleic acid $C_{18}H_{34}O_2$	b p=181—2°C (0.18—0.2 mm.)	$d_4^{18}=0.9106$	$n_D^{20}=1.4390$	197.6 Calc. 198.6	93.7 Calc. 90.1
Elaidic acid $C_{18}H_{34}O_2$	b p=204—5°C (0.4—0.5 mm.) m p=43—4°C	—	—	201.0 Calc. 198.6	88.9 Calc. 90.1
Linoleic acid $C_{18}H_{32}O_2$	b p=193—4°C (0.1 mm.)	$d_4^{18}=0.9248$	$n_D^{20}=1.4685$	201.5 Calc. 200.0	181.5 Calc. 181.4
Linoleic acid $C_{18}H_{30}O_2$	b p=200—1°C (0.4—0.5 mm.)	$d_4^{18}=0.9239$	$n_D^{20}=1.4778$	200.8 Calc. 200.6	265.8 Calc. 273.9
Clupanodonic acid (Japanese Iwashi san)*					
Stearic acid (Kahlbaum) $C_{18}H_{36}O_2$	69—70°C				

was now extracted twice with petroleum ether below b p 50°C. After separating the water layer from the petroleum ether the water solution was acidified with HCl, and again extracted with the petroleum ether. After dehydrating with anhydrous  $Na_2SO_4$ , the petroleum ether solution was evaporated as completely as possible in a high vacuum in  $CO_2$  atmosphere.

The methyl ester was assigned by Sahashi<sup>(10)</sup> as in the following table; its chemical structure is under investigation.

Table II.

	b p/m p	d	$n_D$	Acid value	Iodine value
"Iwashi san" methyl ester	b p=174—5°C (0.018—0.02 mm.)	$d_4^{20}=0.9290$	$n_D^{20}=1.4868$	174.2 (titrated as acid)	340.0 (Rosemund) 338.0 (Wij'smethed)

His remarks on the preparation of the fatty acids mentioned above are summarized as follows:

Oleic acid was prepared by redistilling, using mercury pump, a special preparation which contained no linoleic acid made by E. Merck Company: Eleidic acid was made from Merck oleic acid of German pharmacopoeia by

\* M. Tsujimoto gave the formula  $C_{18}H_{28}O_2$  for clupanodonic acid.

treating it with sodium nitrite and nitric acid ( $d=1.2$ ), it was converted into Pb-salt and then was recrystallized from benzene. After separating as free acid and distilling in order to secure the removal of a trace of Pb, it was recrystallized from alcohol: Linoleic acid and linolenic acid were prepared from soy bean oil and linseed oil respectively according to the method given in "Riken Iho"<sup>(11)</sup>: By saponifying with alcoholic potassium hydroxide, brominating in ether solution, collecting the resulted tetrabromostearic acid (m.p.  $114^{\circ}\text{C}$ ), reesterifying with methyl alcohol containing hydrogen chloride gas, and distilling in a high vacuum; obtaining the free acids by saponifying with alcoholic potassium hydroxide at ordinary temperature, and by distilling in a high vacuum in  $\text{CO}_2$  atmosphere.

From the data of the analysis, it can be recognized that the fatty acids used in this work were 100 per cent pure.

### Results.

The results obtained from feeding were divided into two parts according to the different periods of the experiment:

	A, First part (Chart I—II)	B, Second part (Chart III—VII)
Period	January—April	April—August

During the first period the animal room was maintained at a moderate temperature, average  $70^{\circ}\text{F}$ , and the animals attained growth regularly, whereas for some intervals of the second period, especially in the later stage, it was raised to a temperature as high as  $85-95^{\circ}\text{F}$ , and the growth of some animals was irregular and retarded even when administered with unsaturated fatty acids, and though its improvement was usually expected, sometimes death happened suddenly.

#### A. First Part.

a) The animal group fed on the basal diet and administered with 3 drops of linoleic acid daily (0.078 g. corresponding to about 0.5%) grew healthy; the hair was very fine and lustrous. It was especially noticeable that the eyes were extremely clear and bright (Chart I).

b) The group fed with oleic acid instead of linoleic acid showed the sign of losing hair around the nose and the mouth. First, swelling of the front paws was noticed, and later, slight "scaling", to be described below, appeared on the hind legs. But this symptom seemed to be specific since the growth was continuing at an approximately normal rate (Chart I).

(11) Y. Sahashi: *Ibid*, **10** (1931), 578.

c) The rats reared on the basal diet alone developed a sign of nutritional disorder between the 7th and the 8th week of the experiment. First, the loss of hair around the nose, the mouth; and the eyes took place, and there appeared a tendency to lose the hair on the chest and under arms, sometimes spreading to the back. The period at which baldness occurred, the regions of the body first affected, and the character of the loss of hair varied greatly among individuals, but the areas of baldness were usually symmetrical. The bald areas looked moist and inflamed. Later, there were swelling and hemorrhage of the nose and the mouth. In severe cases; the tip of the tongue and the lips underwent necrosis and dropped off in the manner of gangrene and sometimes inside of the mouth cavity became necrotic. Dermatitis appeared while these symptoms were developing and the exudate dried on the skin, where fine silky hair had matted, formed crusts resembling "scales". In all cases, the front paws were swollen and at times hemorrhage at the joints or necrosis at the end of the front paws appeared, and the hind paws became "scaly". The eyes were swollen and often closed by accumulation of secretion, but there was no change in the cornea as in the case of xerophthalmia due to the deficiency of vitamin A.

Blood urine or occult blood in the urine was not often observed in these rats, although Evans and Lepkovsky<sup>(9)</sup> stressed on hematuria as an important part of the symptoms.

The growth was retarded with the development of the symptoms so far described, then the decline of the body weight followed and the rats were observed to sit in a humped position. When this condition continued, the animals could not live much longer unless they were administered with either linoleic acid or linolenic acid. On healing, a soft fine coating of hair appeared on the denuded areas after a few days, and growth proceeded rapidly and the animal soon resumed a normal appearance.

On post-mortem examination there was no definite macroscopic change found in the internal organs except atrophy of lymphoid system and fatty degeneration of liver in some cases.

The symptoms developed on the fat-free diet seem to be a kind of dermatitis accompanying no suppuration and strikingly resemble pellagra recorded in the literatures and also a disease resulted from high white egg diet reported by Parsons<sup>(12)</sup> in some respects. Some examples are illustrated by the photographs.

Fig. 1 illustrates some of the symptoms produced on fat-free diet. The rat (a) died two days after the photograph had been taken, and the rat (b) died soon after the photograph had been taken. [Refer to (A) and (B) in

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(12) H. T. Parsons: *J. Biol. Chem.*, **90** (1931), 351.

## Chart II].

The rat (a) in Fig. 2 had the symptoms mentioned above, and was suffering from inflammation of chest, swelling of paws, "scaling" of hind legs and pus under arms. This rat lost its weight gradually, and then suddenly from



Fig. 1.

116g to 94g within only two days. When such a rapid decrease occurred, death happened soon. By administering 2 drops of linoleic acid daily, the rat regained its weight, and in a few days pus under arms disappeared and a new skin was formed. Its weight increased to 150 g during the treatment with the acid for 20 days and the rat (b) recovered completely. (Refer to E in Chart II).

Fig. 3 shows the process of recovering of the "scaly" feet developed with fat-free diet. The rat had about the same symptom as the rat in Fig. 2 (a). As soon as 3 drops daily of linoleic acid were administered, the rat renewed its growth, "scaly" feet disappearing: a) before treatment, b) 11th day after starting daily administration of 3 drops of linoleic acid, c) 14th day



(a) 94 g.



(b) 150 g.

Fig. 2.



(a)



(b)

Fig. 3.



(c)



(d)



(e)

Fig. 3.

beef liver. The lesion, however, was cured by administering 2 drops of linoleic acid daily; d) tail lesion and e) complete recovery of the symptom. after same treatment. (Refer to F in Chart III).

In the course of recovery, this rat has shown a tail lesion nearly identical with that on rich egg white diet described by Parsons<sup>(12)</sup>, who claimed that neither 10 drops daily of linseed oil nor commercial lard were effective for cure, but recovered completely when administered with 20 per cent of dried

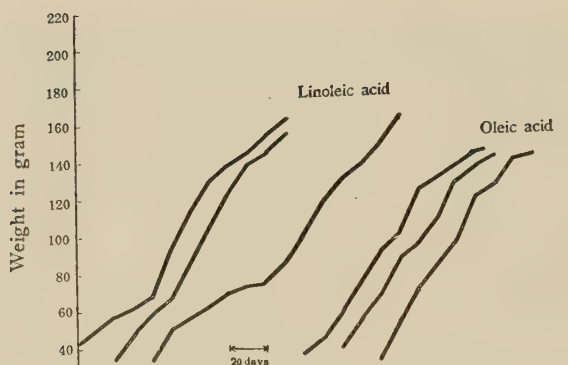


Chart I—Showing the effects on growth of administering 3 drops daily of linoleic acid and of oleic acid.

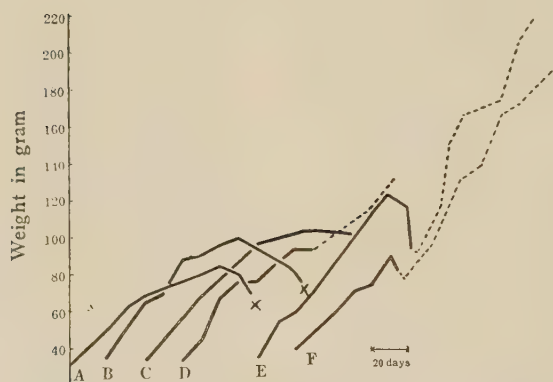


Chart II—Showing the effects on growth of basal diet alone, and the renewed growth and rapid recovery from the deficiency disease by the treatment with linoleic acid.  
× indicates death.

### B. Second Part.

Since linoleic acid had shown a remarkable effect in curing the fat deficiency, a study of the other acids containing 18 carbon atoms, saturated and unsaturated, was undertaken.

a) *Linolenic acid*:- One drop daily of this acid was used. When growth was somewhat retarded, as shown in Chart III a, the dose was increased to 2 drops, but no great improvement could be obtained. This retardation and irregularity, not found with linoleic acid, seemed to be rather

influenced by heat as seen in Chart III *a*.

As in Chart III *b*, it was proved that linolenic acid was almost identical with linoleic acid in curing rats suffering from deficiency of fat (Chart III *a* and *b*).

b) Clupanodonic acid:- One drop of the acid was used daily, but the feeding on this acid was found difficult on account of its characteristic fishy smell and its easy oxidation in the air: the rats ejected the acid; the acid changed to dry, varnish-like mass in the course of a few days unless special precautions were taken to prevent oxidation, and so on. One rat showed a very remarkable growth with nearly a normal appearance, while the other gained weight for a short interval after being fed on the dose; then lost appetite and began to decline and death was preceded by a rapid loss of weight. The bareness of the back, the chest and paws, and around the eyes and the mouth was noticed, but "scaly" feet never occurred on these rats. Diarrhea was the most noticeable feature,

An additional experiment was performed to see the effects of linoleic acid and linolenic acid on the sick animals produced on this dose. Two out of three sick animals were fed with 2 drops of linoleic acid and the third with the same amount of linolenic acid.

Unfortunately, neither of the acids showed any beneficial effects upon these rats and they soon died. This failure in curing might be due to the fact that emaciation and malnutrition advanced too far as they did not much take in the diet; thus this experiment was of little value (Chart IV).

c) Linoleic acid with

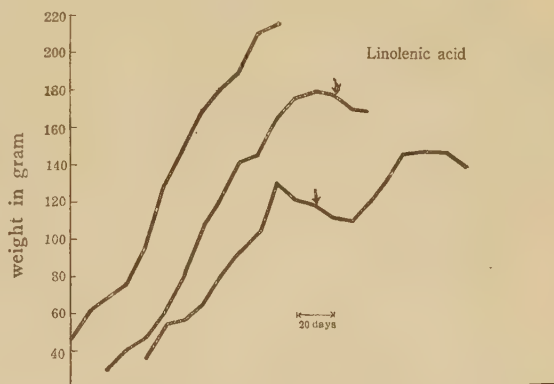


Chart III *a*.—Showing the effects on growth of administering one or two drops daily of linolenic acid. The arrow indicates increase of the acid from one drop to two.



Chart III *b*.—Along with the renewed growth there is rapid recovery from the deficiency disease.

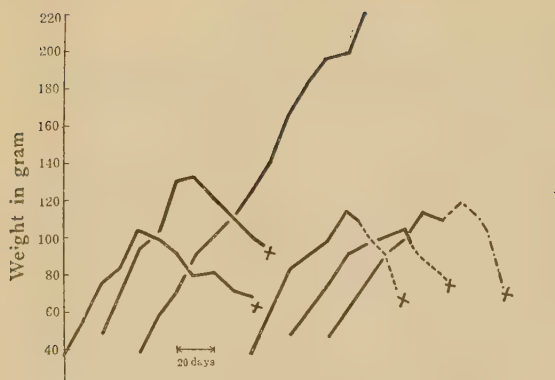


Chart IV.—Showing the effects on growth of administration daily with one drop of clupanodonic acid. X indicates death.

Unfortunately, however, though the rats grew for a short period on this diet, the growth became practically stationary, and the rats gradually lost weight and died except one rat. The symptoms as already mentioned on the fat-free diet were not remarkable but for a tendency to produce incrustation on the tail.

By replacing the oryzanin solution with the yeast extract, the rat soon

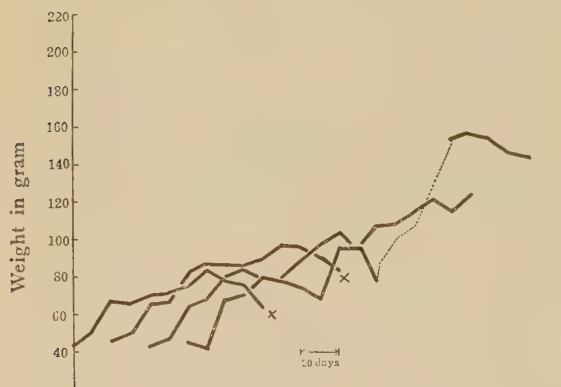


Chart V.—Showing the effects on growth of supplementing with oryzanin solution as vitamin B source instead of yeast extract, and the renewed growth by replacing oryzanin solution with yeast extract. X indicates death.

active oryzanin\* instead of the yeast extract:— This was conducted with the object to determine whether linoleic acid could spare vitamin G as suggested by Hume and Smith<sup>(13)</sup>. Two drops of linoleic acid and one drop of 10% active oryzanin solution daily (each drop corresponding to 4 mg. of the original oryzanin) were used; later, the doses were increased to 3 drops of the acid and 2 drops of oryzanin solution.

gained weight and resumed a normal appearance. When it was changed again to the oryzanin solution, the rat began to lose weight (Chart V).

It seems, therefore, as though fat has no sparing action on vitamin G.

d) Elaidic acid:— 5 rats were reared on the diet containing 0.5 per cent elaidic acid. There were two distinct features appearing in this group: In one case the rats gained weight, while in the

\* I am indebted to Dr. S. Odake for supplying the active oryzanin which cured severe antineuritic pigeons with 4 mg. daily.

The same yeast extract as used in the whole experiments was employed, and its curing power on pellagra rats has been measured previously.

(13) E. M. Hume and H. H. Smith: *Biochem. J.*, **25** (1931), 292.

other they suddenly lost weight. Such a phenomenon also appeared in the experiments of Sahashi<sup>(9)</sup>. The reason for these two different features is not fully understood. In the former, if such is the case, the biological value of elaidic acid seems to be superior to that of oleic acid. None of the rats showed the "scaly" feet. In the latter, however, one rat died though administered with 2 drops of linolenic acid, while the other regained weight (Chart VI).

e) Stearic acid:— The diet containing 0.5 per cent recrystallized stearic acid was used in this experiment. The rats receiving the stearic acid diet exhibited a remarkable growth for some interval, and suddenly lost weight and died. Evans and Lepkovsky<sup>(9)</sup> stated that animals on the stearic acid diet were in poorer condition than those on fat-free diet, and this might be due to the fact that the acid was poorly absorbed (Chart VII).

### Summary.

1. It is confirmed that both linoleic acid and linolenic acid are equally effective in curing a specific deficiency disease produced by rigidly fat-free diets.
2. Oleic acid and elaidic acid induce growth response of rats, but seem to be ineffective in curing sick rats.
3. The rats receiving clupanodonic acid suddenly lose body weight and are not cured with either linoleic acid or linolenic acid.
4. The poor results obtained with stearic acid may be attributed to imperfect absorption.
5. Vitamin B and G are definitely ruled out as a limiting factor in this dietary deficiency.

The author wishes to express her deep gratitude to Professor U. Suzuki

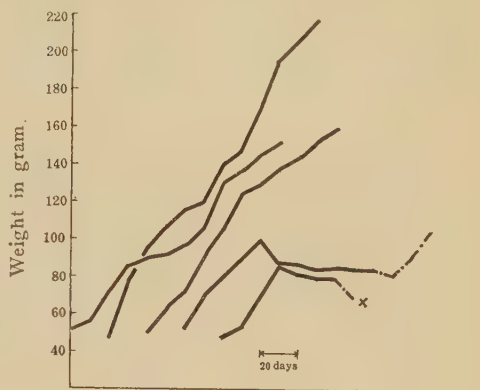


Chart VI.—Showing the effects on growth of adding 0.5 per cent of elaidic acid to the basal diet, × indicates death.

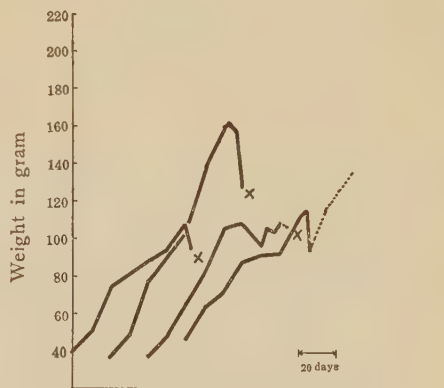


Chart VII.—Showing the effects on growth of adding 0.5 per cent of stearic acid to the basal diet, × indicates death.

for his many helpful suggestions throughout this work and to Dr. W. Nakahara for his invaluable advice on the anatomical examination. She is also indebted to the Keimei Society for financial assistance.

## **Sterilising Action of Acids on Putriferous Bacteria, *Bac. typhosus* and *Vib. cholerae*.**

First Report.—Sterilising action of Mineral Acids.

Sogo TETSUMOTO.

(Received September 19, 1932.)

### **Introduction.**

If we compare sterilising power of the same concentration of reagents, we know that acids have stronger sterilising power than bases or salts generally <sup>(1)</sup> <sup>(11)</sup>. Owing to this fact, there are many studies about sterilising action of acids.

But before Krönig und Paul's fundamental studies about the sterilising action of reagents, many studies seem the observation of acid molecules only. Krönig and Paul said that  $P_H$  are the essential part about sterilising action of acids and that anions and undissociated molecules have very weak or no sterilising power. After this study Bail's, Paul and Birstein's, and many other studies published one after another about this problem.

If we summarise all these studies until now, are as follows:

- (1) Sterilising action of acids depends on  $P_H$  almost totally.
- (2) Anions or undissociated molecules have no sterilising power or have very weak sterilising power.

But according to my many experiments, of course  $P_H$  is the essential part about the sterilising action of acids, following questions arise;

- (1) Are anions and undissociated molecules really weak, or almost no sterilising power?
- (2) How are the sterilising power of each reagent under the important condition of the experiment e. g. (to experience always about certain quantities of the microorganisms having constant resisting power)? This condition is very important and that this has been treated carelessly by many experimenters.
- (3) How are the collation of sterilising action at the same  $P_H$  and at the same normal solution?

(4) Possibility to find out harmless and effective sterilising chemicals and their utilization for drink and diet.

To study and clear up more thoroughly on these questions I performed following experiments. I wish to express my profound thanks to Dr. S. Kojima for his kind leading about my study.

### Experiment.

#### (1) Inoculation of microorganisms.

Taking a sufficient quantity of microorganisms to ascertain their life and death, and then to cause the loss of the toxic action of reagent upon bouillon, I inoculated one platinum loopful of culture: the diameter of the loop was 4 mm., having the diameter of platinum wire 0.6 mm.

#### (2) Mineral acids used.

$\text{HNO}_3$ ,  $\text{HCl}$ ,  $\text{H}_2\text{SO}_4$ ,  $\text{H}_3\text{PO}_4$  (ortho), Chromic acid (anhydride), Osmic acid  
(Osmium tetra oxide),  $\text{HCNO}$ ,  $\text{H}_3\text{BO}_3$ .

#### (3) Species of microorganisms used for the experiments.

Name	gram	Mobility
<i>Staphylococcus pyogenes aureus</i>	+	—
<i>Bacillus typhosus</i>	—	+
<i>Proteus vulgaris</i> Hauser	+	+
<i>Vibrio cholerae</i>	—	+

#### (4) Standard culture media.

For the purpose to keep the constant vital forces and resisting power for *Staph. c. pyog. aur*, *Bac. typhosus*, *Prot. vulgaris*, I used next bouillon and agar.

Standard Bouillon:	Liebig's meat extract	5 g.	} by NaOH a. q. adjusting to PH 7.0
	Peptone	10 "	
	NaCl	5 "	
	H <sub>2</sub> O	1000 c.c.	

Standard agar: With above bouillon made 2.5% agar slant.

For *Vib. cholerae*,

Standard Bouillon:	Liebig's meat extract	5 g.	} by 10% Na <sub>2</sub> CO <sub>3</sub> adjusting to PH 8.0
	Peptone	10 "	
	NaCl	5 "	
	H <sub>2</sub> O	1000 c.c.	

Standard agar: With above bouillon made 2.5% agar slant.

#### (5) Standard resisting power of microorganisms used for experiments.

For 5 days, I repeated 24 hours bouillon cultures of *Staph. c. pyog. aureus*, *Proteus vulgaris* H., *Bac. typhosus* and *Vib. cholerae* with standard bouillon, and fixed their respective vital forces. With pure phenol crystal

(M. P. 40°C) I made watery solution of 1/75, 1/90, 1/100, 1/175 dilution by weight.

According to G. Reddish's method<sup>(13)</sup>, I chose next group among many cultivated microorganisms.

Surviving time (minute)	Phenol solution			
	1/75 dilution	1/90 dilution	1/100 dilution	1/175 dilution
	Staph. coc. pyog.	Bac. typhos.	Prot. vulg.	Vib. chol.
5	+	+	+	+
10	+	±	±	±
15	—	—	—	—

+ ..... alive    — ..... death    ± ..... alive or death,

With these 4 species I made standard agar slant culture every day at 37°C.

#### (6) Time.

From the put instant of the inoculation into bouillon, I set the reaction time as follows:

1 m., 2.5, 5, 10, 15, 20, 30, 45, 60, 90, 2 h., 3, 6, 9, 12, 24, 36, e. t. c.  
m.....minute    h.....hour

#### New Process Tried On Chemical Sterilisation.

G. Reddish's method used for the chemical sterilisation, is as follows:

Reagent 10 c.c. + 24 hours bouillon culture 1 c.c. of microorganism. This process is used generally now. But if we put 1 c.c. of bouillon culture into 10 c.c. of reagent, concentration and chemical characters of reagent will be greatly changed by it. Above all this fact is distinct when reagent is acid solution. For example;

Reagent	Normal	PH	PH after adding 1 c.c. of Bac. typhos. bouillon culture	PH after adding 1 c.c. of Vib. chol. bouillon culture	PH after adding 0.1 c.c. of Bac. typh. bouillon culture	PH after adding 0.1 c.c. of Vib. chol. bouillon culture
HNO <sub>3</sub>	N/100	2.0	2.8—3.0	3.2—3.4	2.1—2.2	2.2—2.4
HCl	"	"	"	"	"	"
H <sub>2</sub> SO <sub>4</sub>	"	"	"	"	"	"
H <sub>3</sub> PO <sub>4</sub>	"	2.1	"	"	"	"
H <sub>2</sub> CrO <sub>4</sub>	"	4.4	5.4—5.6	5.8—6.0	4.6—4.8	4.8—5.0
H <sub>2</sub> OsO <sub>4</sub>	1/200,000	5.6	6.4—6.6	6.6—6.8	5.8	5.8—6.0
H <sub>3</sub> BO <sub>3</sub>	1 Nor.	4.8	5.6—5.8	6.0—6.2	5.0—5.2	5.2—5.4
HNO <sub>3</sub>	1/1,000	3.0	4.0	4.2—4.4	3.2	3.2—3.4
HCl	"	"	"	"	"	"
H <sub>2</sub> SO <sub>4</sub>	"	"	"	"	"	"

H <sub>3</sub> PO <sub>4</sub>	"	3.2	4.4—4.6	4.6	3.6	3.6
H <sub>2</sub> CrO <sub>4</sub>	"	4.8	5.8—6.0	6.0	5.0—5.2	5.0—5.4
H <sub>2</sub> OsO <sub>4</sub>	1/1,000,000	5.6	6.8—7.0	6.8—7.2	5.8	5.8—6.2

(20°C)

Namely if we put 1 c.c. of bouillon culture of *Bac. typhosus* into 10 c.c. of reagent,  $P_H$  of reagent change 0.8—1.2. On *Staph. c. pyog.* and *Proteus vulgar.*, we see also nearly the same results. On *vib. choler.*, the change of  $P_H$  is 1.2—1.6. Even if we put 0.1 c.c. of bouillon culture of microorganism, into 10 c.c. of reagent, the change of  $P_H$  is 0.1—0.4 on *Bac. typhosus*, *Prot. vulgaris* and *Staph. c. pyogenes*, and 0.2—0.6 on *Vib. cholerae*. According to this respect Shimokawa<sup>(1)</sup>, Tsubouchi<sup>(5)</sup> took such process that instead of bouillon culture 1 c.c. they attached microorganism to the silk thread or artificial wild-cocoon silk thread with certain mass and diameter, and put it into reagent. By this method change of reagent will be relatively small. But into such solution as pure dilute acid, silk thread or artificial wild cocoon silk thread will give some change on chemical character of reagent during relatively long time. Krönig and Paul<sup>(8)</sup> adhered suspension of microorganisms to garnets having the certain size, and put these into reagents. By above methods microorganisms will be much near the silk thread or artificial wild-cocoon thread or garnet and will be not homogeneously distributed in reagents.

To avoid these defects, I tried new process; that is as follows:

I put 0.1 c.c. of bacterial suspension to 10 c.c. of reagent with the pipet measuring 1/100 c.c. degree, to forbid nothing to enter into the reagent except bacterial suspension, and to avoid the change of chemical characters or concentration of reagent.

Bacterial suspension:—

Put 2 mg. of colony of 24 hours standard agar slant culture of microorganism into 10 c.c. of the sterilised physiological NaCl solution and make homogeneous suspension.

By this method nothing can enter into reagents except the microorganism, and that total germs of 0.1 c.c. of bacterial suspension are as follows:

Bac. typhosus}	20,000,000 germs
Vib. cholerae}	
Proteus vulgaris Hauser	23,000,000 "
Staph. c. pyogen. aureus	28,000,000 "

The change of  $P_H$  are as follows:

Reagent	Concentration (Normal)	P <sub>H</sub>	P <sub>H</sub> after adding 1 c.c. of Bac. typh. suspension	P <sub>H</sub> after adding 1 c.c. of Vib. chol. suspension	P <sub>H</sub> after adding 0.1 c.c. of Bac. typh. suspension	P <sub>H</sub> after adding 0.1 c.c. of Vib. chol. suspension
HNO <sub>3</sub>	1/100	2.0	2.2	2.2—2.4	2.0	2.0
HCl	"	"	"	"	"	"
H <sub>2</sub> SO <sub>4</sub>	"	"	"	"	"	"
H <sub>3</sub> PO <sub>4</sub>	"	2.1	2.4	2.4	2.1	2.1
H <sub>2</sub> CrO <sub>4</sub>	"	4.4	4.6	4.6—5.0	4.4	4.4
H <sub>2</sub> OsO <sub>4</sub>	1/200,000	5.6	5.8	5.8—6.0	5.6	5.6
H <sub>3</sub> BO <sub>3</sub>	1	4.8	5.0	5.0—5.2	4.8	4.8
HNO <sub>3</sub>	1/1,000	3.0	3.2	3.2—3.4	3.0	3.0
HCl	"	"	"	"	"	"
H <sub>2</sub> SO <sub>4</sub>	"	"	"	"	"	"
H <sub>3</sub> PO <sub>4</sub>	"	3.2	3.4—3.6	3.4—3.6	3.2	3.2
H <sub>2</sub> CrO <sub>4</sub>	"	4.8	5.0—5.2	5.0—5.4	4.8	4.8
H <sub>2</sub> OsO <sub>4</sub>	1/1,000,000	5.6	5.8—6.0	5.8—6.2	5.6	5.6

Namely if I put 0.1 c.c. of suspension of *Bac. typhosus*, or *Vib. cholerae*, into 10 c.c. of reagent, there is almost no change about P<sub>H</sub> of the reagent. On *Staph. c. pyogenes* and *Proteus vulgaris* Hauser, I saw the same result. Performance of the experiment:—

Take 2 mg. from each colonies of 24 hours standard agar slant culture of *Staph. c. pyog. aureus*, *Proteus vulgaris* Hauser, *Bac. typhosus* and *Vib. cholerae*, and put each 2 mg. into 10 c.c. of sterilising physiological NaCl solution and make 4 microorganic suspensions. Make these 4 species of suspensions at 20°C in incubator (difference within 0.5°C). Also keep each series of 10 c.c. taken from reagents at 20°C. Put 0.1 c.c. of the suspension into 10 c.c. of reagent and mixed homogeneously. At the every certain time I inoculate microorganisms from reagents into standard bouillon with the certain platinum loop and cultivate them 48—72 hours in incubator at 37°C. Alive or death of microorganisms are determined by the turbidity of standard bouillon culture. When the result is doubtful I repeat the experiment many times, and then I ascertained by morphological investigations and the agglutination test with rabbit's immune serum of each microorganisms.

Results are following tables:

Experimental results:—

Table No. 1. Weight% and P<sub>H</sub> at *N*/100, *N*/1000, of HNO<sub>3</sub>, HCl, H<sub>2</sub>SO<sub>4</sub>, H<sub>3</sub>PO<sub>4</sub> (ortho), Cromic acid, HCNO, 1 Nor. of H<sub>3</sub>BO<sub>3</sub>, *N*/100,000, *N*/1,000,000 of Osmic acid.

Table No. 2. 3. Sterilising action at *N*/100, *N*/1000, except boric acid, Boric acid.....1 Nor.

Table No. 4. Sterilising action of dilute chromic acid and extremely dilute solution e. g.  $N/100,000$ ,  $N/1,000,000$  of osmic acid.

Table No. 5. Relation between  $P_H$  and normal concentration of mineral acids which affect on sterilisation.

Table No. 6. Sterilising action of anion of each mineral acids.

To ascertain the sterilising action of anion of mineral acids, 1 made alkaline salts having the same anion of each acids. For Staph. coc. pyogen, Prot. vulgar. and Bac. typhos.  $N/100$  of alkaline salts of each acids and  $N/100,000$  of alkaline osmium salt, 1 nor. of alkaline boric acid salt, are used. For Vib. chol.  $N/1,000$  of each alkaline salts,  $N/1,000,000$  of alkaline salt of osmic acid,  $N/5,000$  of alkaline salt of chromic acid, 1 nor. of alkaline salt of boric acid are used. Each of K and Na salts give nearly the same results, so 1 denote the results of K-salt only.

Table No. 1. Weight % and  $P_H$  of reagents.

Reagents	Molecul. weight	Concent. (Normal)	$P_H$	Weight %	Concent. (Normal)	$P_H$	Weight %
$HNO_3$	63.018	1/100	2.0	0.063	1/1,000	3.0	0.0063
HCl	36.468	"	"	0.037	"	"	0.0037
$H_2SO_4$	98.016	"	"	0.049	"	"	0.0049
$H_3PO_4$	98.064	"	2.14	0.033	"	3.2	0.0033
$CrO_3$	100.01	"	4.4	0.050	"	4.8	0.0050
$OsO_4$	255.0	1/100,000	5.4	0.000128	1/1,000,000	5.6	0.000013
HCNO	43.021	1/100	4.4	0.043	1/1,000	4.6	0.0043
$H_3BO_3$	61.924	1 Nor.	4.8	2.064			

Table No. 2. Sterilising action at  $N/100$  solution.

Name of microorg.	Staph. c. pyog.						Prot. vulgar.						Bac. typhos.						Vib. chol.					
	15	20	30	45	60	90	1	2.5	5	10	15	20	2.5	5	10	15	20	30	1	2.5	5	20	30	45
Surviving period						m						m					m						m	
HNO <sub>3</sub>	+	+	+	-	-	-	+	+	+	±	-	-	+	+	+	±	-	-	-	-	-	-	-	-
HCl	+	+	+	-	-	-	+	+	+	±	-	-	+	+	+	±	-	-	-	-	-	-	-	-
H <sub>2</sub> SO <sub>4</sub>	+	+	+	-	-	-	+	+	+	±	-	-	+	+	+	±	-	-	-	-	-	-	-	-
H <sub>3</sub> PO <sub>4</sub>	+	+	+	±	-	-	+	+	+	+	-	-	+	+	+	+	+	-	±	-	-	-	-	-
H <sub>2</sub> CrO <sub>4</sub>	+	+	-	-	-	-	+	±	-	-	-	-	+	±	-	-	-	-	-	-	-	-	-	-
H <sub>2</sub> OsO <sub>4</sub>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HCNO	+	+	-	-	-	-	+	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-
Il <sub>3</sub> BO <sub>3</sub>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	±	-
Control	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

$H_3BO_3$ .....1 Normal

m.....minute

+.....alive

-.....death

±.....alive or death

Table No. 3. Sterilising action at  $N/1000$  solution.

	Staph. c. pyog.						Prot. vulgar.						Bac. typhos.						Vib. choler.					
	3	6	9	12	24	36 <sup>h</sup>	1	2	3	6	9	12	2	3	6	9	12	24 <sup>h</sup>	2.5	5	10	15	20	30 <sup>m</sup>
Surviving period																								
HNO <sub>3</sub>	+	+	+	±	-	-	+	+	+	-	-	-	+	+	+	-	-	-	+	+	+	±	-	-
HCl	+	+	+	±	-	-	+	+	+	-	-	-	+	+	+	-	-	-	+	+	+	±	-	-
H <sub>2</sub> SO <sub>4</sub>	+	+	+	±	-	-	+	+	+	-	-	-	+	+	+	-	-	-	+	+	+	±	-	-
H <sub>3</sub> PO <sub>4</sub>	+	+	+	+	±	-	+	+	+	+	-	-	+	+	+	+	-	-	+	+	+	+	+	-
H <sub>2</sub> CrO <sub>4</sub>	+	+	±	-	-	-	+	+	-	-	-	-	+	±	-	-	-	-	-	-	-	-	-	-
H <sub>2</sub> OsO <sub>4</sub>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HCNO	+	+	-	-	-	-	+	+	-	-	-	-	+	±	-	-	-	-	±	-	-	-	-	-
H <sub>3</sub> BO <sub>3</sub> (1 Normal)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	±
Control	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Table No. 4. Sterilising action of dilute Cromic acid and extremely dilute solution of Osmic acid.

	Conc. (Normal)	PH	Staph. c. pyog.					Prot. vulg.			Bac. typhos.				Vib. choler.				
			2.5	5	10	15	20 <sup>m</sup>	1	2.5	5	10 <sup>m</sup>	2.5	5	10	15 <sup>m</sup>	10	15	20	30
H <sub>2</sub> CrO <sub>4</sub>	1/2,000	5.2													+	-	-	-	-
"	1/5,000	5.4														+	+	+	+
H <sub>2</sub> OsO <sub>4</sub>	1/100,000	5.4	+	+	+	-	±	-	-	-	+	+	-	-	-	-	-	-	-
"	1/200,000	5.6	+	+	+	+	-	+	+	-	+	+	±	-	-	-	-	-	-
"	1/500,000	"													-	-	-	-	-
"	1/1,000,000	"													+	-	-	-	-
Control			+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Table No. 5. Sterilising action of the same PH of mineral acids, such as HNO<sub>3</sub>, HCl, H<sub>2</sub>SO<sub>4</sub>, H<sub>3</sub>PO<sub>4</sub>.

PH 2.0	Staph. c. pyogen.				prot. vulgar.				Bac. typhos.				Vib. Choler.			
	15	20	30	45 <sup>m</sup>	2.5	5	10	15	5	10	15	20 <sup>m</sup>	1	2.5	5	10
HNO <sub>3</sub>	+	+	+	-	+	+	±	-	+	+	±	-	-	-	-	-
HCl	+	+	+	-	+	+	±	-	+	+	±	-	-	-	-	-
H <sub>2</sub> SO <sub>4</sub>	+	+	+	-	+	+	±	-	+	+	±	-	-	-	-	-
H <sub>3</sub> PO <sub>4</sub>	+	+	+	-	+	+	±	-	+	+	±	-	-	-	-	-

P <sub>H</sub> 3.0	Staph. c. pyogen.				Prot. vulgar.				Bac. typhos.				Vib. chol.			
	6	9	12	24 <sup>h</sup>	2	3	6	9 <sup>h</sup>	3	6	9	12 <sup>h</sup>	10	15	20	30 <sup>m</sup>
HNO <sub>3</sub>	+	+	±	-	+	+	-	-	+	+	-	-	+	±	-	-
HCl	+	+	±	-	+	+	-	-	+	+	-	-	+	±	-	-
H <sub>2</sub> SO <sub>4</sub>	+	+	±	-	+	+	-	-	+	+	-	-	+	±	-	-
H <sub>3</sub> PO <sub>4</sub>	+	+	±	-	+	+	-	-	+	+	-	-	+	±	-	-

Table No. 6. Sterilising action of anions of mineral acids.

	Staph. c. pyog.				Prot. vulgar.				Bac. typhos.				Vib. chol.			
Surviving period	m	15	m	d	d	2.5	5	10	d	m	m	d	m	15	20	30
KNO <sub>3</sub>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
KCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
K <sub>2</sub> SO <sub>4</sub>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
K <sub>3</sub> PO <sub>4</sub>	+	+	+	+	+	+	+	±	+	+	+	+	+	+	+	+
K <sub>2</sub> CrO <sub>4</sub>	+	+	+	±	-	+	+	±	-	+	+	±	-	+	+	±
K <sub>2</sub> OsO <sub>4</sub>	+	+	-	-	-	+	+	-	-	+	-	-	+	+	-	-
KCNO	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
K <sub>3</sub> BO <sub>3</sub>	+	+	+	+	±	+	+	±	-	+	+	+	+	+	+	+
Control	+	+	±	-	-	+	+	±	-	+	+	±	-	+	+	±

m.....minute

h.....hour

d.....day.

## Summary.

- (1) Sterilising action of mineral acids such as HNO<sub>3</sub>, HCl, H<sub>3</sub>PO<sub>4</sub> is determined by P<sub>H</sub> of each solution, and has no relation about kind of acids.
- (2) Anions of strong mineral acids have no sterilising action.
- (3) There is no special relation between the value of molecular weight of mineral acids and sterilising action.
- (4) On sterilising action of chromic acid and HCNO, P<sub>H</sub> concerns a little.
- (5) Sterilising action of dilute chromic acid and HCNO, such as N/100 are almost due to the action of undissociated acid molecule.
- (6) The violent sterilising action of Osmic acid is chiefly due to the action of its anion and P<sub>H</sub> concerns a little.
- (7) Sterilising action of Boric acid is chiefly due to P<sub>H</sub> and the sterilising action of it is very feeble.

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